Molecular Motors of ESX-Type Secretion Systems

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Abstract

Tuberculosis is an enormous global health problem. Despite decades of research, the mechanism(s) by which *Mycobacterium tuberculosis* (Mtb) mediates virulence remains incompletely understood. The ESX-1 secretion system is critical for Mtb to survive and cause disease *in vivo*, but its primary function and mechanism of action are unclear. The many inherent challenges of working with this slow-growing pathogen often limit the experimental approaches that can be used to address these questions. Thus, we have developed a model system in the nonpathogenic bacterium *Bacillus subtilis* to study ESX-type secretion systems. Here, we demonstrate that the *B. subtilis yuk* operon encodes an ESX-type secretion system responsible for the secretion of YukE. Additionally, we demonstrate that the *yuk* system is active in *B. subtilis* during conditions of nutrient deprivation and is required for normal biofilm formation. Interestingly, this is similar to our findings that the Mtb ESX-1 system plays dual roles in protein secretion and modulating cell wall integrity.

One defining feature of all ESX loci is the presence of an FtsK/SpoIIIE family ATPase. Interestingly, these ATPases have a domain structure unique to ESX-associated ATPases, where each protein contains multiple (2-3) enzymatic domains. We used our *B. subtilis* system to dissect the mechanism of action of this unique class of motor proteins. We find that the *yuk*-encoded ATPase YukBA dimerizes to form a hexamer of enzymatic subunits that are differentially required for secretion. Strikingly, we find a unique requirement for rotational symmetry in the nucleotide binding activity of the subunits.

Finally, we compared the energy requirements of the Mtb ESX-1 system and the *B. subtilis yuk* system. We find that these systems have some overlapping ATPase requirements for protein secretion and cell wall integrity/biofilm formation, suggesting that there is a conservation of function among ESX-type systems. We also find that some ATPase domains are differentially required for function between these two systems, which we postulate is due to the split protein architecture of the ESX-1-encoded ATPases. Together, these findings highlight the power of using a *B. subtilis* model system to understand the function and mechanism of action of ESX-type secretion systems.

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A manuscript including the work discussed in Chapter 2 is currently in preparation. The work in this chapter was performed in collaboration with co-authors Laura Huppert and Briana Burton (Harvard University). The experiments displayed in Figures 2-2, 2-4, 2-5 and Supplemental Figure 2-1 were performed by Laura Huppert. Alejandra Garces, Dave Sarracino and Mike Chase performed the mass spectrometry.

A manuscript including the work discussed in Chapter 3A is currently in preparation. This work was performed in collaboration with co-authors Laura Huppert and Briana Burton (Harvard University). The dissertation author performed all experiments in this chapter and is the author of the manuscript in preparation. We thank Briana Burton and the Burton lab for their assistance with the FPLC system.

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The work discussed in Chapter 4 is unpublished. The experiment displayed in Figure 4-1 was performed with Krish Atmakuri, a former post-doctoral fellow in the Fortune laboratory. The experiment in Figure 4-3 was performed with Laura Huppert of the Burton lab.

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CHAPTER 1: Introduction

1.1 Bacterial protein secretion systems

Pathogens have evolved a variety of mechanisms to manipulate the host, and often these mechanisms are dependent upon the interaction of a secreted bacterial effector with a particular host cell target (Finlay 1997). These substrates, whether they are proteins, lipids, or small molecules, must first gain access to the proper host cell environment. This requires the transport of the substrate from the bacterial cytoplasm, across the cell membrane(s) and cell wall, where the substrate ultimately remains attached to the bacterial cell surface and is exposed to the extracellular environment, is secreted into the extracellular milieu, or is translocated directly into the host cell cytosol. Thus, bacterial secretion systems are essential mediators of virulence for many pathogens.

Bacterial secretion systems are also required for a diverse array of homeostatic functions. Thus, bacteria have evolved a conserved set of mechanisms and systems to mediate the export of substrates out of the cell. The most common mechanisms for protein secretion across the bacterial inner membrane are the general secretory Sec pathway and the twin arginine translocation TAT pathway. Found in all bacteria, these systems transport unfolded and folded proteins, respectively. Substrates of the Sec pathway are recognized by an N-terminal signal sequence, which is cleaved as part of the export process. Likewise, substrates of the TAT system are targeted for secretion by a similar N-terminal signal sequence which includes a double arginine motif.

For Gram-negative bacteria, protein secretion requires the substrate to traverse both the inner plasma membrane and an outer membrane. To deal with this second barrier, Gram-negative species have evolved a number of strategies to move substrates from the cytoplasm to the extracellular environment. These specialized secretion systems, numbered type I thru type VI,

can either be a one step process, where the secretion machinery spans from the bacterial cytoplasm through the entire cell envelope, or a two step process, first requiring a substrate to be translocated across the inner membrane by the Sec or TAT translocon (Finlay 1997).

Secretion by type III and type IV systems adds another layer of complexity, as these systems transport substrates across a third lipid bilayer, the host cell plasma membrane. The type III secretion machine is a complex structure which terminates in a needle-like complex that projects from the bacterial surface (Galan 1999). Upon contact with a eukaryotic host cell, a highly regulated process ensues, resulting in the translocation of protein effectors through the needle's inner channel and into the host cell. Type IV secretion systems have a wide range of functions including DNA conjugation, DNA uptake, DNA release, protein secretion, and the injection of virulence factors into eukaryotic host cells (Cascales 2003). Similar to type III systems, the type IV secretion machine relies on a surface protruding pilus structure to deliver protein and DNA effectors to the host cell.

Protein secretion by Gram-positive bacteria has historically been thought to be less complex in comparison to their Gram-negative counterparts, as substrates have only one bacterial cell membrane to cross. Thus, Gram-positive organisms were not thought to require specialized secretion systems, although the over simplification of this belief has become increasingly apparent over the past decade. Often, a typical Sec or TAT signal sequence is sufficient to direct the secretion of a protein across the cytoplasmic membrane and peptidoglycan layer into the extracellular environment. Some proteins are targeted to the cell wall and remain anchored in the peptidoglycan layer through a hydrophobic targeting motif (LPXTG) at their C-terminus (Finlay 1997).

Mycobacterial species, which are neither truly Gram-negative nor Gram-positive, represent a unique set of challenges in terms of protein secretion. In addition to a classical inner membrane and a thick layer of peptidoglycan common to Gram-positive bacteria, mycobacteria have an additional impermeable barrier made up of mycolic acids (often called the mycomembrane), and more distally, a layer of lipids and glycolipids (Brennan 1995). While mycobacteria use the well-conserved Sec and TAT systems to secrete proteins across the inner membrane, it is still unclear how bacterial proteins traverse the waxy coat of this bacterium to reach the extracellular milieu. The genus *Mycobacterium* is composed of both nonpathogenic and pathogenic species, including human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Given that the lifestyle of mycobacterial pathogens involves intimate interactions between the bacterium and the host cell, it would seem advantageous for these species to encode a type III- or type IV-like secretion system, as is the precedent set by many intracellular pathogens. But, comprehensive genomic analysis revealed no such homologous system. Within the last decade, the identification and characterization of a specialized secretion system present in mycobacteria has provided new insight into the complexities and variety of bacterial protein secretion systems.

1.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb), the causative agent of the disease tuberculosis (TB), is a devastating human pathogen and worldwide health problem. In 2009, the World Health Organization reported over 9 million new cases and 1.7 million deaths (World Health Organization 2010). Although this is a treatable disease, the lack of an effective vaccine, the prevalence of TB in the immunocompromised HIV-infected population, and the recent emergence of highly drug resistant strains presents a continuing challenge for global control.

Upon inhalation, Mtb takes up residence in the host lung to either cause active disease or develop into a latent infection. Active disease is characterized by robust bacterial replication and a counteracting host immune response, which together lead to the clinical manifestations of the disease. Conversely, a latent infection is asymptomatic and can persist for decades. Though the details of latency are poorly understood, Mtb has evolved mechanisms to promote its own persistence and viability, while the host is able to locally contain and control the infection. These bacteria retain the ability to transition into an active disease state at a later time, often upon compromise of the host immune system, commonly as a consequence of disease or aging.

The interaction between a host and pathogen is dynamic and complex. As a successful human pathogen, Mtb actively avoids clearance by the immune system to ensure its own survival. Within the lung, Mtb resides in macrophages, modulating the function of these professional phagocytic cells to create a protective niche for itself. The bacterium enters the macrophage through the binding of various phagocytic receptors, including complement receptors and mannose receptors (Ernst 1998). Upon phagocytosis, the bacterium finds itself in an early phagosome. Normally, the phagosome goes through a maturation process involving fusion with the lysosome to create an environment hostile to the invading microbe, characterized by low pH, reactive oxygen species, and lysosomal enzymes. Intracellular pathogens have evolved a variety of mechanisms designed to stop this process and avoid being killed. Mtb specifically targets an early step in the process of vesicle maturation by inhibiting fusion of the bacteria-containing phagosome with lysosomes (Armstrong 1971). Additionally, Mtb is able to minimize acidification of the vacuole by excluding proton-pumping ATPases from the compartment in which it resides (Sturgill-Koszycki 1994). In the end, this active ability of Mtb to modulate the macrophage and its antimicrobial defense mechanisms results in the creation of a protective niche in which to replicate and persist.

1.3 ESX-1 identification

Though the mechanisms by which Mtb promotes its own survival are complex, it was long ago recognized that secreted proteins play an integral role in the host-pathogen interaction, as secreted proteins were identified as being the most potent T-cell antigens during an Mtb infection. In an effort to understand the immune response to this pathogen, proteins in short term culture filtrates of *in vitro* grown Mtb cultures were analyzed. Among secreted proteins, the most highly immunogenic antigens were found to be proteins with molecular masses between 3-9 kDa and 25-31 kDa (Andersen 1993). Purification and characterization experiments identified the dominant low molecular weight antigen to be the protein subsequently named 6 kDa early secretory antigenic target, or ESAT-6 (Andersen 1995, Sørensen 1995). Subsequently, ESAT-6 was found to be co-transcribed with a second gene encoding for a secreted protein later named 10 kDa culture filtrate protein, or CFP-10 (Berthet 1998). Both ESAT-6 and CFP-10 are small, 100 amino acid proteins with no homology to known proteins. Most interestingly, both proteins lack a canonical signal sequence for the general secretory pathway. These findings suggested that Mtb may use a specialized secretion system to export ESAT-6 and CFP-10 during infection.

In silico analysis, made possible by the completion of the whole genome sequence of the most well characterized laboratory strain of Mtb, H37Rv, revealed that ESAT-6 is part of a larger gene family consisting of 14 members. Interestingly, five of these ESAT-6 family proteins are encoded within genetic loci with a highly conserved organization (Cole 1998, Tekaia 1999). These five gene clusters, originally named ESAT-6 cluster regions 1-5, are the result of genetic duplication events (Gey van Pittus 2001). In addition to the pair of ESAT-6 and CFP-10 family proteins encoded within each locus, these loci also contain a conserved set of genes encompassing various protein families, including PE/PPE proteins, ABC transporters, ATP-binding proteins, large multi-pass transmembrane proteins, and integral membrane proteins of

unknown function (Tekaia 1999). The features and conservation of these gene clusters led multiple groups to hypothesize that these loci may encode a secretion apparatus responsible for the transport of ESAT-6 and CFP-10 family proteins across the inner cell membrane and/or the cell wall (Cole 1998, Tekaia 1999, Gey van Pittius 2001, Pallen 2002).

Concurrent to the genetic and proteomic characterization of Mtb, studies of virulence highlighted the importance of one specific ESAT-6 gene cluster, region 1, in the pathogenesis of this bacterium. Clues to identifying the major virulence determinants of Mtb came from the study of *Mycobacterium bovis* Bacille Calmette-Guerin (*M. bovis* BCG). The serial passage of the virulent bovine pathogen *M. bovis* over multiple decades resulted in an attenuated strain, *M. bovis* BCG, which has been used as a live attenuated vaccine against tuberculosis for nearly a century. Through genetic comparison of *M. bovis* BCG, virulent *M. bovis* and Mtb H37Rv, it was suggested that the primary attenuating deletion of the *M. bovis* BCG strain was the loss of a 9.5-kb segment of DNA designated RD1 (Mahairas 1996). Included within this genomic region are the genes for the abundantly secreted antigens ESAT-6 and CFP-10. The hypothesis that the loss of RD1 was the major attenuating deletion of *M. bovis* BCG was later experimentally supported, as the reintroduction of the RD1 locus into *M. bovis* BCG significantly restored the virulence of this strain in mice, though not fully to the levels of wild-type *M. bovis* (Pym 2002). More directly, when the precise RD1 deletion from *M. bovis* BCG was recreated in pathogenic Mtb H37Rv, it resulted in an attenuation of virulence in both macrophages and mice (Lewis 2003).

The role of RD1 in virulence was again highlighted by experiments to comprehensively define the requirements for Mtb growth *in vitro* and *in vivo*. Transposon mutations within this genetic region did not affect growth of the bacterium *in vitro*, but these genes were found to be essential for growth in both mice and macrophages (Sassetti 2003a, Sassetti 2003b, Rengarajan 2005). The identification of multiple RD1-encoded genes as being individually required for virulence

added support to the hypothesis that these genes act together as a functional unit to directly mediate Mtb pathogenesis.

The 9.5 kb genetic region defining RD1 is part of a larger genetic locus initially termed ESAT-6 gene cluster region 1. This locus was later more precisely defined to include genes Rv3864-Rv3883 (*espE –mycP*$_1$) and was named ESAT-6 system-1, or ESX-1 (Brodin 2004). In 2009, a systematic nomenclature for defining the components of ESX loci was adopted (Figure 1-1) (Bitter 2009). To keep within these guidelines, the ESX system components referred to in this document will from here on follow this accepted nomenclature.

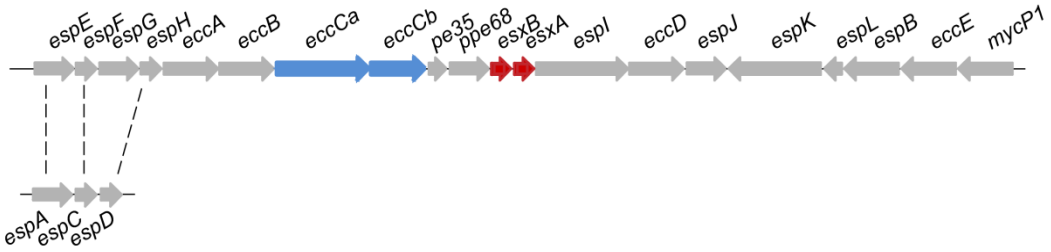


Figure 1-1. Genetic organization of the *Mycobacterium tuberculosis* ESX-1 locus. Schematic depicting the ESX-1 locus (top) and *espA* operon (bottom). Genes highlighted in blue (*eccCa* and *eccCb*) encode the FtsK/SpoIIIE family ATPases relevant to this dissertation. Genes highlighted in red encode the conserved substrates EsxA and EsxB.

The genes of ESX-1 were hypothesized to play a direct role in pathogenesis by encoding a specialized secretion system responsible for the translocation of virulence effectors. To begin to test this hypothesis, multiple groups took similar genetic approaches to test the effects of individual ESX-1 gene disruptions on secretion and virulence. Mutations, either through transposon insertion or gene deletion, in *eccCa*, *eccCb*, *esxA*, *esxB*, *espI*, and *eccD* were found to abolish the secretion of EsxA and EsxB (formerly ESAT-6 and CFP-10) (Stanley 2003, Guinn 2004). To directly test the hypothesized association between ESX-1 secretion and virulence,

individual ESX-1 mutants were assayed in macrophage and mouse models of infection. Secretion defective strains with mutations in *eccCa*, *eccCb*, or *eccD* showed delayed growth kinetics, a less robust inflammatory response, and decreased overall virulence in mice (Stanley 2003). Similarly, Guinn et al. showed that mutants lacking individual ESX-1 genes phenocopied an RD1 mutant strain in mice and macrophage infections, demonstrating defects in growth, inflammation, and cytotoxicity (2004). Together, these data support the prediction that the ESX-1 locus encodes for the components of a secretion system that work together to translocate the locus-encoded proteins EsxA and EsxB. Furthermore, the secretion of these proteins is coupled to Mtb pathogenesis, as any strain capable of secreting EsxA and EsxB shows wild-type levels of growth and pathogenicity in models of infection, while any ESX-1 mutant defective for secretion is attenuated for virulence. The fact that the disruption of a single gene attenuates the bacterium to the same extent as deleting the entire locus again provides evidence that the genes within this locus work together as a functional unit.

1.4 Components and mechanism of the ESX-1 secretion system

To better understand how ESX-1 functions to mediate virulence, we can first aim to understand the mechanism by which ESX-1 assembles and exports proteins. By combining functional and structural predictions with protein-protein interaction studies, insight into the molecular mechanism of the ESX-1 secretion system can be gained. To date, six ESX-1 substrates have been identified. The originally identified substrates, EsxA and EsxB, form a tight, heterodimeric complex and are dependent on one another for stability and secretion (Renshaw 2002). EspA, EspB and EspC are also substrates of the ESX-1 system (Fortune 2005, Xu 2007, McLaughlin 2007, MacGurn 2005). *espA* and *espC*, along with a third gene, *espD*, are encoded within a second gene cluster unlinked to the ESX-1 locus. Unusually, these five substrates are mutually dependent upon one another for secretion, as deletion of any one substrate abolishes the secretion

of all other substrates (Fortune 2005). The molecular basis for this codependence is unclear; these proteins may be secreted as part of a multimeric complex, or alternatively, these proteins may be structural components of the secretion system, and the stability of the transport machine could require the presence of each component (Fortune 2005). Lastly, a DNA-binding transcription factor, EspR, is also an ESX-1 secreted substrate (Raghavan 2008). EspR binds and activates transcription of the *espACD* operon and is itself secreted, thereby downregulating *espA* expression, creating a negative feedback loop to regulate secretion.

The ESX-1 substrates are hypothesized to be translocated across the inner cell membrane through a pore created by the multipass transmembrane protein EccD, though this prediction has not been experimentally demonstrated. The mechanism by which the ESX-1 substrates are translocated across the mycobacterial cell wall is currently unclear. The energy for substrate translocation is thought to be provided by the locus-encoded proteins EccCa and EccCb. EccCa contains N-terminal transmembrane domains, which presumably anchor the protein into the cytosolic face of the inner membrane, and a single ATPase domain of the FtsK/SpoIIIE family. EccCb contains two like ATPase domains. EccCa and EccCb are predicted to act as a single functional unit, because in other Mtb ESX loci, these proteins are encoded for by a single open reading frame, which was divided into two separate genes during the evolution of the ESX-1 genetic duplication (Gey van Pittus 2001). This prediction is supported by the demonstration that EccCa and EccCb interact *in vitro*, by a yeast two-hybrid assay (Stanley 2003).

An *in vitro* yeast two-hybrid system also identified an interaction between the C-terminus of EccCb and the substrate EsxB (Stanley 2003). A subsequent study identified the last 7 amino acids of EsxB as being both necessary and sufficient for the interaction with EccCb (Champion 2006). These 7 amino acids are part of the unstructured C-terminal tail of EsxB, which remains exposed upon formation of the EsxA:EsxB dimer. Therefore, it is believed that the EsxB signal

sequence targets the EsxA:EsxB dimer for secretion. Similarly, the C-terminus of the substrate EspC was also shown to be required for interaction with another ESX-1-encoded ATPase, EccA (Champion 2009). EccA is a cytosolic protein of the AAA ATPase family, which has been shown to possess functional enzymatic activity and to self hexamerize *in vitro* (Luthra 2008). The C-terminal sequences of EspC and EsxB are functionally distinct, and unlike the EsxB C-terminal sequence, the EspC sequence is necessary, but not sufficient for secretion (Champion 2009).

1.5 Two models of ESX-1 function

While the identification of the ESX-1 secretion system as a major determinant in the pathogenesis of Mtb has been well recognized, the mechanism by which this system mediates virulence is still poorly understood. Traditionally, ESX-1 was believed to mediate virulence through the secretion of a small number of protein substrates directly into the host cell. While this is an enticing model, a mechanism by which the known ESX-1 substrates might act on the host cell is unclear, as none of these proteins contain conserved functional domains that would suggest a mechanism by which the proteins would act as virulence effectors.

There has been some evidence to suggest that EsxA has pore forming abilities and may act as an effector of host cell cytolysis, though this idea remains controversial. Using an artificial membrane system Hsu et al. showed that purified EsxA could disrupt a lipid bilayer (2003). Also using an *in vitro* system, de Jonge et al. demonstrated a strong association between purified EsxA and liposomes, which resulted in the destabilization and lysis of the liposomes (2007). But, when EsxA is present in a 1:1 complex with EsxB, these proteins no longer associate with liposomes. The *in vivo* relevance of these findings are questionable, as structural studies have demonstrated

the extreme stability of the EsxA:EsxB dimer, leading multiple groups to conclude that the biologically active form of these proteins is as a complex (Renshaw 2002, Lightbody 2008).

Further complicating the model that the primary mechanism of ESX-1-associated virulence is mediated by the pore-forming ability of EsxA is the conservation of ESX-1 and *esxA* in nonpathogenic bacterial species. This includes nonpathogenic mycobacteria, such as the soil-dwelling species *Mycobacterium smegmatis*, as well as various Gram-positive species, both pathogenic and nonpathogenic (Gey van Pittius 2001, Pallen 2002). It is unclear how a conserved virulence effector such as EsxA would benefit organisms representing such diverse bacterial lifestyles. It is possible that EsxA has evolved functions specific to the needs of each organism or, the ESX system of each organism may secrete a different set of substrate proteins, specific to the environmental niche in which it resides.

Alternatively, ESX systems may have a common function representing a more basic, homeostatic process. It is known that the mycobacterial cell wall is a complex structure, and proper assembly and maintenance of this structure is critical for the survival of the bacterium, both *in vitro* and *in vivo*. Indeed, the connection between colony morphology, often a gross readout of cell wall composition, virulence, and ESX-1 was long ago recognized. Steenken et al. isolated mycobacterial colonial variants and demonstrated a strong association between variations in morphology and differences in virulence in various animal models (1934, Petroff 1930). Interestingly, the attenuated strains Mtb H37Ra and *M. bovis* BCG were originally isolated from populations of virulent Mtb H37Rv and *M. bovis*, respectively, based on a change in colony morphology (Calmette 1927, Steenken 1938). It is hypothesized that this change in morphology coincided with the loss of ESX-1 function and the resulting attenuation of these strains (Pym 2002, Frigui 2008). When the ESX-1 locus was reintroduced into *M. bovis* BCG both virulence

and colony morphology reverted to that of *M. bovis* and Mtb H37Rv (Pym 2002). These observations suggest that ESX-1 plays a role in modulating the mycobacterial cell wall.

In validation of the above observations, Garces et al. recently demonstrated that ESX-1 is a critical mediator of cell wall integrity, and it is hypothesized that it is through this function that ESX-1 mediates virulence, independent of protein secretion (2010). This suggests that the primary target of ESX-1 is the mycobacterial cell wall. It is possible that an ESX-1 substrate, perhaps the substrate protein EspA, modifies a component of the cell wall, or alternatively, ESX-1 may be responsible for the translocation of cell wall components. The loss of cell wall integrity that occurs due to ESX-1 mutations may detrimentally affect the host-pathogen interaction, leading to the observed virulence defects of the strains in the above study.

Many details of the biology of the ESX-1 system remain to be elucidated. However, the inherent difficulties of working with pathogenic Mtb have thus far hindered our ability to manipulate this pathogen in the robust manner necessary to address the detailed mechanistic questions required for the understanding of ESX-1 function on a molecular level. But, ESX-type secretion systems are conserved throughout Gram-positive bacteria, many of which are nonpathogenic, thereby providing an opportunity to gain insight into the function(s) and mechanism of action of ESX-type secretion systems using a more tractable bacterial model.

1.6 Gram-positive ESX-type secretion systems

The identification of ESX-type secretion systems in other bacterial species stemmed from the increasing recognition of the importance of EsxA-like proteins in mycobacteria, which led to the use of *in silico* methods to search for EsxA homologues in other bacteria. Homologues were first identified in the phyla actinobacteria (high G+C Gram-positive bacteria) (Gey van Pittius 2001),

and have more recently been identified in firmicutes (low G+C Gram-positive bacteria) (Pallen 2002) and chloroflexi (Sutcliffe 2011). The recognized presence of ESX-type systems outside of mycobacteria led to the proposal that these systems be identified as a new, distinct class of alternative secretion systems, thereby deserving to be added to the conventional nomenclature of bacterial secretion systems. Thus, ESX-type systems became termed "type VII secretion systems" (Abdallah 2007), although this nomenclature is not universally accepted.

Conserved components of ESX-type secretion systems

Two proteins are conserved among all ESX-type systems, a small EsxA-like protein and a predicted ATPase of the FtsK/SpoIIIE family (Pallen 2002). The sequence homology between EsxA-like proteins is low, but these proteins all share some defining characteristics, including a size of approximately 100 amino acids, the presence of a WXG amino acid motif, a helical protein structure, and the lack of an N-terminal signal sequence. These features led to the categorization of these proteins as "WXG100" family proteins (Pallen 2002). The ESX-associated FtsK/SpoIIIE family ATPases contain multiple (2-3) ATPase domains. This is in contrast to canonical members of this protein family, which have a single ATPase domain. In fact, among ATPases of the FtsK/SpoIIIE family, the presence of multiple enzymatic domains is found solely in ATPases encoded within ESX loci. Thus, the close genetic proximity of a multi-FtsK/SpoIIIE domain containing ATPase with a WXG100 protein-encoding gene serves as a genetic marker for ESX-type systems.

While all ESX loci encode at least one WXG100 protein and an FtsK/SpoIIIE family ATPase, there is a large amount of variability in the additional genes that make up each locus, both in number and homology. Many of the genes conserved among mycobacterial ESX loci are not present in other species. Likewise, some genes are conserved solely among firmicutes and others

among actinobacteria. Thus, it is unclear whether ESX-type systems share a conserved function and/or a conserved mechanism of action.

FtsK/SpoIIIE family ATPases

The ATPases associated with ESX loci are of the FtsK/SpoIIIE protein family. ATPases of this family are conserved throughout bacteria and are predominately involved in the translocation of DNA and proteins through membrane-spanning pores (Iyer 2004). FtsK/SpoIIIE proteins are important in the processes of cell division, sporulation and DNA conjugation, as well as others. The founding members of this family, FtsK of *E. coli* and SpoIIIE of *B. subtilis*, are responsible for the translocation of double stranded chromosomal DNA from the mother to the daughter cell compartment during cell division and sporulation, respectively. FtsK coordinates chromosome segregation and chromosome dimer resolution during cell division to ensure the equal partitioning of genetic material between the mother and daughter cell (Begg 1995, Aussel 2002). SpoIIIE is required for the segregation of chromosomal DNA from the mother cell to the prespore during the asymmetric cell division process of sporulation (Wu 1994). FtsK and SpoIIIE achieve these functions by translocating along the DNA in an ATP-dependent manner, resulting in the pumping of DNA from one compartment to the other (Bath 2000, Aussel 2002).

Clues to the molecular mechanism by which FtsK/SpoIIIE family ATPases couple ATP hydrolysis to substrate translocation first came from structural studies of AAA+ family ATPases. Many members of this superfamily, including RecA and DnaB, have been shown to function as oligomers, specifically hexamers (Iyer 2004). Additional structural insight came from the crystal structure of the R388 plasmid-encoded DNA translocase TrwB. TrwB, which is similar to FtsK/SpoIIIE family proteins, is essential for the transfer of single stranded DNA during bacterial conjugation (Gomis-Ruth 2001). The crystal structure of TrwB revealed a homohexameric ring structure with a central channel large enough to accommodate a single strand of DNA, leading to

the hypothesis that ATP hydrolysis powers the translocation of a single DNA strand through the hexameric ring into the recipient cell during mating. Structural data on FtsK followed and demonstrated that FtsK similarly forms ring-shaped hexamers with a central channel large enough to allow the passage of double stranded DNA (Massey 2006).

Studies of non-mycobacterial ESX-type secretion systems

It remains to be determined whether ESX-type secretion systems are part of a general virulence strategy of Gram-positive pathogens. The ESX-type secretion systems of the pathogens *Staphlococcus aureus*, *Bacillus anthracis*, and *Listeria monocytogenes* are currently being investigated (Burts 2005, Garufi 2008, Way 2005). While the *S. aureus* ESX-type system is required for the virulence of this pathogen in mouse models of infection, the *L. monocytogenes* system is not required for virulence. Additionally, while the secretion of one or more EsxA-like proteins has been demonstrated in each system, there are differential requirements for the secretion of this substrate among species. For example, the secretion of EsxA and EsxB in *S. aureus* is dependent upon the conserved FtsK/SpoIIIE family ATPase, but the ATPase of this family is dispensable for the secretion of *B. anthracis* EsxB.

The function(s) ESX-type secretion systems in nonpathogenic bacteria is even more unclear. To date, the ESX-type system of only one nonpathogenic bacterium, *Streptomyces coelicolor*, has been studied. In this system, the secretion of an EsxAB heterodimer is dependent upon at least two genes within the locus, one of which is an FtsK/SpoIIIE family ATPase (Akpe San Roman 2010). Interestingly, the deletion of *esxAB* causes a sporulation defect resulting in spore chains with an abnormal wrinkled morphology, a phenotype that is reminiscent of the altered colony morphology associated with the loss of the ESX systems from mycobacteria.

Bacillus subtilis yuk operon

B. subtilis is one of the most widely studied Gram-positive model organisms. In addition to the ease in which this bacterium can be manipulated in the laboratory, there is a wealth of genetic and biochemical techniques and tools available that can be used to probe any number of biologic questions. For these reasons, we have chosen to use *B. subtilis* to develop a tractable model system in which to study the function and mechanism of action of ESX-type secretion systems.

A locus predicted to encode an ESX-type secretion system *B. subtilis* was recognized almost a decade ago (Pallen 2002), but this prediction has yet to be experimentally validated. The *B. subtilis yuk* operon encodes for five genes: *yukE*, *yukD*, *yukC*, *yukBA*, and *yueB*. YukE is the predicted substrate of this potential system, as it is homologous to the Mtb EsxA protein. YukBA is a predicted FtsK/SpoIIIE family ATPase with three ATPase domains. The remaining genes of the *yuk* operon are not homologous to known ESX-associated proteins. Bioinformatic analysis predicts *yukD* to encode a putative bacteriocin, but whether this prediction is functionally relevant is unknown (de Jong 2006). YukC is a predicted membrane-associated protein of unknown function. YueB is a surface-exposed, membrane-bound protein containing six transmembrane domains which has been shown to be essential for the irreversible binding of the bacteriophage SPP1 to the bacterium (São-José 2004).

1.7 Dissertation aims

It is currently unclear what commonalities exist between ESX-type secretion systems and whether these systems retain a conserved function in all species. While all systems studied to date have been shown to be responsible for the secretion of an EsxA-like substrate, the functional consequences of this action are unknown. This family of secretion systems was originally identified because of its role in Mtb virulence, but the conservation of ESX-type systems in

nonpathogenic bacterial species, and the finding that these systems are not universally required for the virulence of ESX-encoding pathogenic bacteria, suggests that these systems may play an important role in the bacterium itself. Additionally, the conservation of an FtsK/SpoIIIE family ATPase with a domain structure unique to ESX-type systems suggests that these systems may share a common mechanism of action. Thus, the continued study of ESX-type secretion systems in various bacterial species will provide insight as to how these secretion systems work, and with this knowledge we aim to gain understanding of the mechanism by which the ESX-1 system of Mtb mediates the virulence of this devastating human pathogen.

In this dissertation, we aim to address questions of ESX function and mechanism by combining studies of the Mtb ESX-1 secretion system with the development of a tractable model system in the nonpathogenic bacterium *B. subtilis*. Chapter 2 describes the development of the B. *subtilis* model to study the ESX-type secretion system of this bacterium, encoded for by the *yuk* operon. We begin to characterize this system by assessing the functions and genetic requirements of the *yuk* system. In Chapter 3A, we determine the requirements for each ATPase domain of YukBA in powering *yuk* secretion. Additionally, we use the *B. subtilis* model to dissect the mechanism of action of this unique class of motor proteins. In Chapter 3B, we test the energy requirements for ESX-1-mediated secretion in Mtb. Lastly, in Chapter 4, we determine the energy requirements for ESX-mediated cell wall functions in each system, allowing for a comparison of the ATPase requirements for secretion and cell wall interactions of both the Mtb and *B. subtilis* ESX-type systems.

CHAPTER 2: Characterization of the *Bacillus subtilis* ESX-type *yuk* secretion system

2.1 Introduction

The ability to transport proteins, DNA, lipids and small molecules across the hydrophobic barrier created by the cell membrane is essential to the survival of every bacterium. Central to this process are bacterial secretion systems, which direct the substrate translocation required for a diverse array of processes, from maintaining homeostasis to mediating virulence. In addition to the general secretory pathway, mediated by the Sec system, and the folded protein secretion pathway, mediated by the TAT system, bacteria have evolved alternative secretion systems to mediate the secretion of dedicated subsets of substrates. These specialized systems are often nonessential, but can be required for bacterial growth or survival under certain conditions. To date, six classes of specialized secretion systems, designated type I-type VI, have been identified and characterized in Gram-negative bacteria. More recently, a novel class of secretion systems unique to Gram-positive bacteria was identified, and later termed type VII secretion systems (Abdallah 2007). The first of these alternative secretion systems was identified in the human pathogen *Mycobacterium tuberculosis* (Mtb).

This secretion system, designated the ESX-1 secretion system, is a critical mediator of Mtb virulence; loss of this system significantly attenuates the bacterium *in vivo* (Stanley 2003, Guinn 2004). Despite the importance of this system, the mechanism by which ESX-1 mediates virulence is not clearly understood. ESX-1 has been hypothesized to mediate virulence through the secretion of protein effectors that act on specific targets in the macrophage. To date, six secreted substrates have been identified, including the first identified protein antigens EsxA and EsxB, as well as EspA, EspB, EspC and EspR (Sorenson 1995, Berthet 1998, Fortune 2005, MacGurn 2005, Xu 2007, McLaughlin 2007, Raghavan 2008). While EsxA is thought to act as a pore-forming toxin, the function of most of these secreted proteins remains unknown (Hsu 2003, de Jonge 2007). More recently, it has been demonstrated that ESX-1-mediated virulence is

independent of protein secretion, and virulence is dependent upon ESX-1-mediated maintenance of cell wall integrity (Garces 2010).

Many questions remain to be answered about the functions and mechanism of the ESX-1 secretion system and ESX-type secretion systems more generally. However, the many inherent challenges of working with Mtb often limit the questions that can be posed. Using *in silico* methods to search for *esxA*-like genes, ESX-type secretion systems have been identified throughout Gram-positive bacterial species, including various actinobacteria (high G+C Gram-positive bacteria) (Gey van Pittius 2001, Pallen 2002), firmicutes (low G+C Gram-positive bacteria) (Pallen 2002), and chloroflexi (Sutcliffe 2011). Within ESX-loci, the genes encoding an *esxA*-like substrate protein and an FtsK/SpoIIIE family ATPase are conserved across all species. The conservation of these key components of the secretion system suggests a conserved mechanism of action. This provides an opportunity to use model bacterial systems to gain insight into how these secretion systems work. While the ESX-type secretion systems of other human pathogens including *Staphlococcus aureus*, *Bacillus anthracis*, and *Listeria monocytogenes* are currently being investigated, we sought to develop a tractable model system using the nonpathogenic, soil-dwelling bacterium *Bacillus subtilis* (*B. subtilis*). This easily manipulated model organism provides a wealth of experimental tools and biologic understanding which we can use to parse the structure and function of this family of secretion systems.

It has been bioinformatically predicted that the *B. subtilis yuk* operon may encode an ESX-type protein secretion system (Pallen 2002). The *yuk* operon encodes five genes, *yukE*, *yukD*, *yukC*, *yukBA*, and *yueB*, which are thought to be under the control of a single promoter (Figure 2-1A). YukE is a small, WXG100-motif containing protein homologous to the secreted substrate EsxA of Mtb, and YukBA is homologous to the ESX-1-encoded FtsK/SpoIIIE-like ATPases EccCa-EccCb. The remaining genes of the *yuk* operon are not homologous to known ESX-associated

proteins. YukD is predicted to encode a small polypeptide, and one study identified *yukD* as a putative bacteriocin-encoding gene by bioinformatic analysis, but this prediction has not been addressed experimentally (de Jong 2006). YukC is predicted to be a single-pass transmembrane protein of unknown function. YueB is a transmembrane protein with six transmembrane segments, and studies have demonstrated that YueB is a phage receptor for the SPP1 bacteriophage (São-José 2004). It is common for phage to utilize exposed protein domains as docking sites, thus, it is likely that YueB has an additional function(s) that is independent of its role as a phage receptor.

In this study, we demonstrate that the *yuk* operon encodes a secretion system responsible for the secretion of the EsxA homologue, YukE. Additionally, we find that the *yuk* system plays a role in biofilm formation in a manner that is independent of YukE secretion.

2.2 Results

YukE is secreted by *B. subtilis*

All ESX-type secretion systems that have been studied to date have been shown to secrete at least one protein homologous to the prototypic ESX-1 substrate EsxA. In *B. subtilis*, this protein is encoded for by *yukE*. Therefore, we first tested whether YukE is secreted into the culture supernatant of *B. subtilis* as predicted. Wild-type cultures of the *B. subtilis* laboratory strain PY79 were grown in rich culture media to mid-exponential phase, at which time whole cell pellet and culture supernatant were collected for analysis. Proteins were separated on an SDS-PAGE gel and assayed for YukE secretion by immunoblot using an antibody generated against full length YukE. YukE was detected in the pellet and supernatant of wild-type *B. subtilis*, but absent from a Δ*yukE* strain (Figure 2-1B).

Genetic requirements for YukE secretion

If the genes of the *yuk* locus encode proteins that come together to form a secretion system, then the loss of any gene in the locus should abolish protein secretion. Indeed, in all ESX-type systems that have been assessed, secretion of the EsxA-like substrate is dependent upon at least some genes encoded within the surrounding genetic locus (Stanley 2003, Guinn 2004, Burts 2005, Garufi 2008, Akpe San Roman 2010). To test the genetic requirements for YukE secretion, we created individual deletion mutants in which we replaced each of the *yuk* genes with an antibiotic resistance cassette via double crossover recombination, generating Δ*yukD*, Δ*yukC*, Δ*yukBA* and Δ*yueB*. Because the *yuk* genes are organized in an operon, we reinserted the *yuk* promoter (P*yuk*) after the antibiotic resistance cassette in each knockout strain to drive expression of the remaining downstream operon genes (diagrammed in Figure 2-1A). We verified that deletions within the *yuk* locus do not result in a growth defect or loss of fitness through growth curves and competition experiments (data not shown, Supplemental Figure 2-1).

To determine whether the genes of the *yuk* operon are required for YukE secretion, we tested the expression and secretion of YukE in each deletion mutant. While YukE is present in the pellet of each deletion strain, YukE was not detected in the culture supernatants of any of the four mutant strains (Figure2-1B). To demonstrate the specificity of these results, a complementing construct containing a *myc*-tagged copy of the corresponding gene, fused to an inducible promoter, was inserted at an ectopic chromosomal locus in each knockout strain. The expression of each construct was verified by immunoblot with an anti-Myc antibody (data not shown). As seen in Figure 2-1B, YukE secretion was fully restored in the Δ*yukD* and Δ*yukBA* strains upon expression of *yukD-myc* and *yukBA-myc*, respectively. Although complementation of Δ*yukC* with *yukC-myc* and Δ*yueB* with *yueB-yueC-myc* did not complement YukE secretion to wild-type levels, a restoration of YukE secretion can be seen in an overexposed immunoblot. Together, these data demonstrate that YukE secretion is dependent upon each gene within the *yuk* operon and this

supports the hypothesis that the *yuk* locus encodes a functional secretion system responsible for the secretion of YukE.

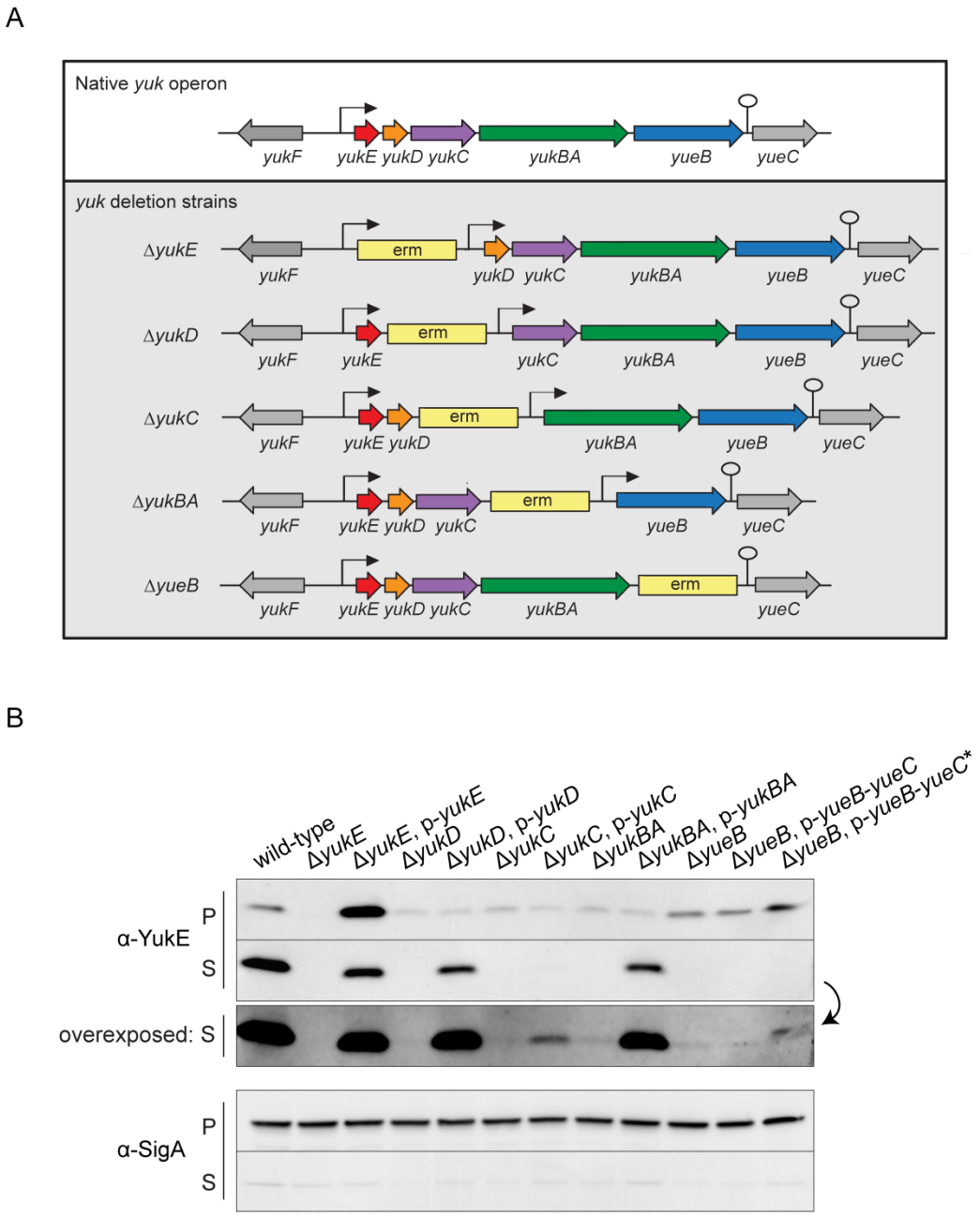


Figure 2-1. *B. subtilis yuk* locus and genetic requirements for YukE secretion.

Figure 2-1 (continued). (A). Schematic depicting the native *yuk* locus (white background) and *yuk* deletion strains used in this study (shaded background). The predicted promoter of the *yuk* operon (*yukE-yueB*) is indicated with a black arrow and the predicted terminator is indicated with a black circle. For each deletion strain, a *yuk* gene was replaced with an antibiotic resistance cassette (erm); the *yuk* promoter (black arrow) was inserted after the antibiotic resistance cassette to drive expression of the remaining downstream operon genes. (B). Immunoblot analysis of cell pellet (P) and culture supernatant (S) of wild-type, *yuk* deletion, and corresponding complemented strains. Samples from cultures grown in LB media were separated by SDS-PAGE under reducing conditions and analyzed by immunoblot with an α-YukE antibody. A Δ*yukE* strain demonstrates antibody specificity. An antibody to SigmaA was used as a lysis and loading control. Asterisk (*) in far right-hand lane indicates overloading of sample 1.5x that of all other lanes. The α-YukE supernatant blot is shown in two exposures; overexposed blot allows visualization of faint bands. Data are representative of at least three independent experiments.

The *yuk* operon is upregulated under nutrient poor growth conditions

The function(s) of ESX-type secretion systems in nonpathogenic bacteria is not known. To begin to address this question, we first sought to determine under which growth conditions the *yuk* operon is expressed. To follow expression from the *yuk* promoter (P*yuk*), we created two *lacZ* transcriptional fusion reporter strains in wild-type *B. subtilis*. This reporter allows us to assay the level of expression from the *yuk* promoter through colorometric β-galactosidase (β-gal) assays. In one strain, the *lacZ* reporter gene was integrated at the endogenous start site of the *yuk* operon (ΩP*yuk-lacZ*). In a second strain, we integrated a P*yuk-lacZ* construct at an ectopic integration site (amyE::P*yuk-lacZ*).

To test whether *yuk* expression varies in response to nutrient availability, we compared the expression of *lacZ* in nutrient rich media (LB) versus defined, nutrient poor media (MC). As seen in Figure 2-2, expression from the *yuk* promoter is 2-fold higher in defined media as compared to nutrient rich media. These results suggest that the *yuk* operon may be important for a bacterial response triggered by nutrient deprivation.

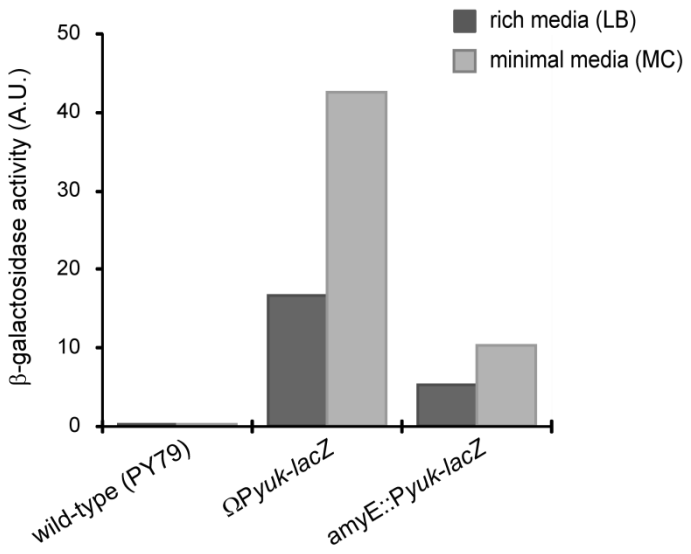


Figure 2-2. The *yuk* operon is upregulated in minimal media.
Expression from the *yuk* promoter (P*yuk*) was measured using P*yuk-lacZ* transcriptional fusions; promoter activity was monitored by quantitative β-galactosidase assays. The P*yuk*-lacZ reporter was integrated at the start of the endogenous *yuk* operon (ΩP*yuk-lacZ*) and at an ectopic integration site (amyE::P*yuk-lacZ*). Strains were grown in rich media (LB) or minimal media (MC). Data are representative of three independent experiments. AU: abritrary units.

YukE is the dominant dedicated substrate of the *yuk* secretion system

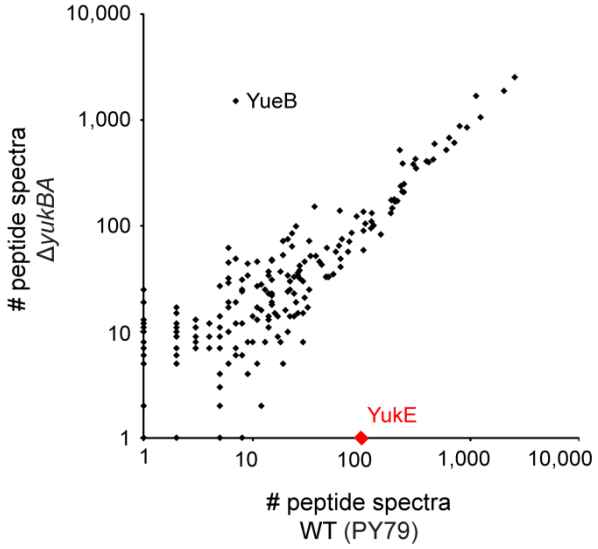
To gain insight into possible function(s) of the *yuk* system, we sought to determine whether there are additional secreted proteins dependent upon the *yuk* system for secretion. In some ESX-type systems, substrates have been identified which are not linked to the primary genetic locus encoding for the secretion system (Fortune 2005, Garufi 2008). Therefore, we sought to globally identify potential *yuk* substrates by using a quantitative proteomics approach to compare the proteins secreted by a wild-type strain versus a strain lacking a functional *yuk* secretion system. Using quantitative mass spectrometry, we compared the culture supernatants of wild-type, Δ*yukBA*, and Δ*yukBA*, p-*yukBA-myc* strains grown in minimal media, as this growth condition was suggested to be functionally relevant based on the upregulation of *yuk* expression. As predicted from our immunoblot analysis, we detected abundant YukE in the culture supernatants of the wild-type laboratory strain; furthermore, YukE was secreted in a *yukBA*-dependent manner

(Figure 2-3). Interestingly, no other proteins were identified that fit the profile of a potential *yuk* substrate; present in wild-type culture supernatants, absent in Δ*yukBA*, and restored upon complementation. These data suggest that YukE is the dominant dedicated substrate of the *yuk* secretion system, though we cannot rule out the possibility that there are other substrates of this system which are secreted under different growth conditions.

A

Strain	WT (PY79)	Δ*yukBA*	Δ*yukBA*, p-*yukBA*$_{myc}$
# YukE peptides	95	0	116

B



C

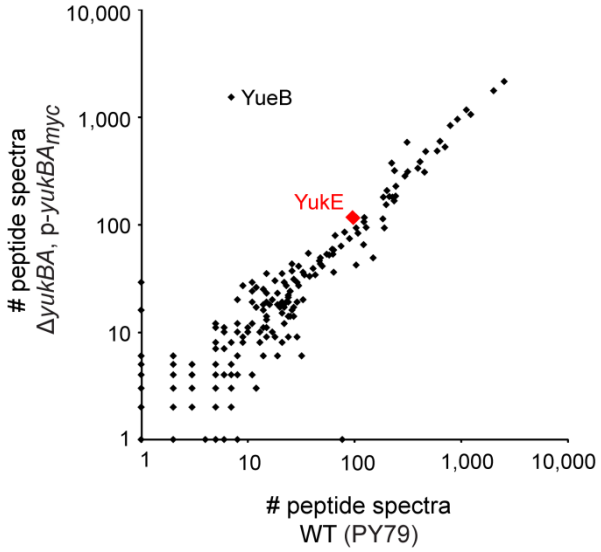


Figure 2-3. YukE is the only protein whose secretion is solely dependent upon YukBA.

Figure 2-3 (continued). (A). Number of YukE-specific peptide spectra detected in the culture supernatants of the indicated strains. (B). and (C). The relative abundance of proteins in the culture supernatants from wild-type compared to Δ*yukBA* (B) or the complemented strain Δ*yukBA*, p-*yukBA-myc* (C) grown in minimal media. Each point corresponds to a specific *B. subtilis* protein. Protein abundance was determined by spectral count analysis; spectral count data are combined totals from three biologically independent samples for each strain. Where no spectra were identified, an arbitrary value of 1 was assigned. Red diamonds indicate YukE; change in YueB abundance is a result of the method used to construct the Δ*yukBA* and Δ*yukBA*, p-*yukBA-myc* strains.

The *yuk* operon is actively expressed in the undomesticated strain *B. subtilis* 3610

We have found that the *yuk* operon is more highly expressed under nutrient limiting growth conditions as compared to nutrient rich conditions. Nutrient depletion is known to trigger a variety of highly regulated responses by *B. subtilis*, such as entrance into stationary phase, induction of sporulation, and biofilm formation. Thus, the upregulation of P*yuk* in minimal media may represent a role for the *yuk* system in one of these processes.

We did not find the *yuk* deletion strains to have defects in stationary phase growth or gross sporulation defects, as compared to a wild-type strain (data not shown). We next tested whether the *yuk* operon plays a role in biofilm formation. *B. subtilis* can respond to stress by forming highly complex, 3-dimensional, multicellular communities known as biofilms. While laboratory adapted *B. subtilis* strains, such as PY79, can form biofilms, undomesticated *B. subtilis* strains can form particularly robust and elaborate biofilm structures (Branda 2001). To address whether the *yuk* secretion system plays a role in biofilm formation, we first sought to test the level of *yuk* expression in the undomesticated strain *B. subtilis* 3610. To do so, we integrated the P*yuk-lacZ* reporter construct into a wild-type 3610 strain, both at the endogenous *yuk* locus (3610, ΩP*yuk-lacZ*) and at an ectopic integration site (3610, amyE::P*yuk-lacZ*). We find the *yuk* promoter to be more highly expressed in the 3610 strain as compared to the PY79 laboratory adapted strain, with levels of expression 2-3 fold higher (Figure 2-4). Additionally, in the 3610 strain, P*yuk* is upregulated in minimal media as compared to rich media.

These results demonstrate that the yuk operon is expressed in the undomesticated 3610 strain, but the above experiments were performed using bacterial cultures grown in liquid media under standard shaking culture conditions. To test whether the *yuk* operon is actively expressed under biofilm-inducing conditions, we spotted the 3610 P*yuk-lacZ* reporter strain on solid biofilm-inducing MSgg media also containing the colormetric substrate X-gal (Branda 2001). We find the *yuk* promoter to be expressed within the biofilm, with the most active expression seen around the central ring of the biofilm and in the actively-dividing cells around the perimeter (Figure 2-4). Thus, the *yuk* operon is expressed in *B. subtilis* 3610 under both standard laboratory and biofilm-inducing growth conditions.

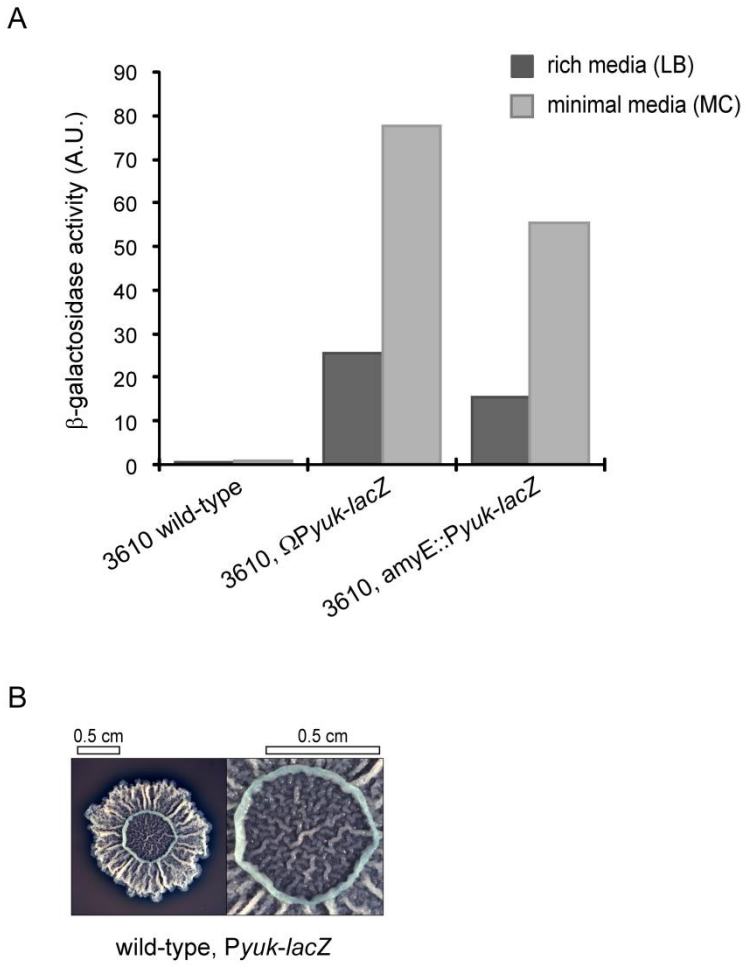


Figure 2-4. Expression from the *yuk* promoter in the undomesticated strain *B. subtilis* 3610. (A). Expression from the *yuk* promoter (P*yuk*) was measured using P*yuk-lacZ* transcriptional fusions integrated into the wild-type undomesticated *B. subtilis* 3610 strain. The P*yuk*-lacZ reporter was integrated at the start of the endogenous yuk operon (ΩP*yuk-lacZ*) and at an ectopic integration site (amyE::P*yuk-lacZ*). Strains were grown in broth culture with rich media (LB) or minimal media (MC); promoter activity was monitored by quantitative β-galactosidase assays. Data are representative of three independent experiments. AU: arbitrary units. (B). A *B. subtilis* 3610 strain containing an integrated P*yuk-lacZ* reporter (amyE::P*yuk-lacZ*) was grown on MSgg agar to induce biofilm formation. Media contained X-gal to allow for visualization of *yuk* expression (blue color). Left panel: entire biofilm; right panel: close up view of biofilm center. Scale bar: 0.5 cm, indicated for each view.

Normal biofilm architecture is dependent upon YukBA

To investigate the functional consequences of disrupting the *yuk* system on biofilm formation, we created Δ*yukE*, Δ*yukD*, Δ*yukC*, and Δ*yukBA* deletion strains in the 3610 background. Each strain

was then plated on solid, biofilm-inducing media and the resulting biofilms were compared to a biofilm produced by wild-type 3610. Knocking out *yukE*, *yukD*, or *yukC* did not alter the appearance of the biofilm as compared to wild-type 3610 (Figure 2-5). Interestingly, biofilms formed by the Δ*yukBA* strain had an altered morphology as compared to wild-type; this defect is most pronounced in the center of the biofilm. The expression of a *yukBA* complementing construct restored the biofilm to an appearance similar to that of wild-type. These results suggest a biofilm-specific function for the *yuk* secretion system that is independent of YukE secretion. Biofilm formation may require the secretion of an additional yet-to-be identified substrate, or the role of the *yuk* system may be secretion independent.

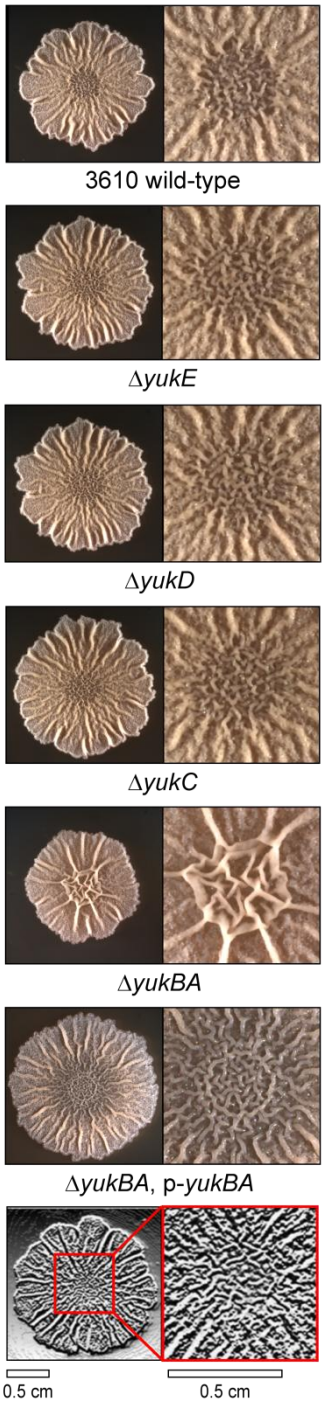


Figure 2-5. Loss of *yukBA*, but not *yukE*, results in a defect in biofilm formation.
B. subtilis 3610 wild-type and *yuk* knockout strains were grown on MSgg agar to induce biofilm formation. As diagrammed in the bottom image, left panels: entire biofilm; right panels: close up view of biofilm center. Scale bar: 0.5 cm, indicated for each view.

2.3 Discussion

We have developed a model system in which to study the function and mechanism of the ESX-type *yuk* secretion system of *B. subtilis*. We have demonstrated that YukE is a secreted protein, as predicted by its homology to the secreted virulence factor EsxA of Mtb. YukE secretion is dependent upon each of the four other proteins encoded within the *yuk* operon, and we have found YukE to be the only dedicated substrate of this secretion system. These results suggest that the *yuk* operon encodes a *bona fide* ESX-type secretion system.

ESX-type secretion systems are conserved throughout pathogenic and nonpathogenic species. It is currently unclear what the primary function of these systems is and whether ESX-type secretion systems share a conserved function(s). All ESX-type systems studied to date have been shown to be responsible for the secretion of a conserved EsxA-like protein substrate, but these proteins do not have an obvious effector function, and it is unclear how the secretion of a single conserved substrate could be beneficial to bacterial species representing such a wide range of lifestyles and environmental niches.

Studies of the ESX-1 system of Mtb have demonstrated that this system mediates virulence not through the secretion of the known ESX-1 protein substrates, but rather virulence is dependent upon a not yet understood role of ESX-1 in maintaining bacterial cell wall integrity (Garces 2010). Interestingly, we have found that the *yuk* secretion system is also important in a fundamental aspect of *B. subtilis* biology, the formation of biofilms. In the absence of the ATPase YukBA, biofilm architecture of the undomesticated *B. subtilis* 3610 strain is altered. This defect is particularly apparent in the center region of the biofilm. The central ring of the biofilm is also the area we found to most highly express the *yuk* operon, suggesting that the *yuk* system may be required for a function specific to cells in this region of the biofilm community.

The requirement for the *yuk* system in biofilm formation is independent of YukE secretion. It is possible that the *yuk* secretion system secretes additional substrate(s) required for this process, and that these substrates are only secreted under biofilm-inducing conditions and thus were not identified in our proteomic analysis of *B. subtilis* supernatants. Alternatively, the *yuk* system may play a role in biofilm formation that is independent of protein secretion. Together, these results suggest that in addition to being responsible for the secretion of EsxA-like substrates, ESX-type secretion systems may share a conserved function in modulating the bacterial cell wall.

2.4 Materials and Methods

Culture of *B. subtilis* and preparation of whole cell pellets and culture supernatants

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman 1983) or, where indicated, the undomesticated strain 3610 (Branda 2001). Strains were maintained in Miller LB broth (Acros, New Jersey, USA) or on Difco Miller LB agar (BD, Sparks, MD). When appropriate, antibiotics were included in the growth media as follows: 100 μg/mL spectinomycin, 5 μg/mL chloramphenicol, 5 μg/mL kanamycin, 10 μg/mL tetracycline, and 1 μg/mL erythromycin plus 25 μg/mL lincomycin (mls). Where indicated, strains were grown in the defined media *B. subtilis* Medium for Competence (MC) (Cutting 1990).

For analysis of protein expression and secretion, mid-log bacterial cultures started from a single colony were back diluted and normalized to an OD600 of 0.02 in LB media and grown 2 hours at 37^{o}C, at which time 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce protein expression. Cultures were grown an additional 2 hours. Cell pellets were collected by centrifugation at 4,000 rpm for 20 minutes and resuspended in lysis buffer (20 mM Tris·HCl pH 7.5, 10 mM EDTA, 1 mM PMSF, 10 μg/ml DNase I, 100 μg/ml RNaseA, 1 mg/mL lysozyme) and incubated at 37^{o}C for 10 minutes. SDS sample buffer (Novex 2x Tricine SDS sample buffer,

Invitrogen) was added and samples were heated to 95°C for 15 minutes. Culture supernatants were filtered though a 0.2 μM filter to remove unlysed cells. Following the addition of a protease inhibitor cocktail (Complete Mini, EDTA-free tablets, Roche, Mannheim, Germany) the supernatants were concentrated by precipitation with 10% tricholoric acid (TCA), resuspended in SDS sample buffer and heated to 95°C for 15 minutes.

Strain construction

General methods for molecular cloning and strain construction were performed according to published protocols (Sambrook 2006). Schematic of *yuk* deletion mutants can be seen in Figure 2-1. Where indicated, the *yuk* promoter was inserted downstream of the knockout construct to drive expression of downstream operon genes. As the *yuk* promoter has not been previously characterized, we used the 417 base-pair sequence directly upstream of *yukE*. We confirmed that this promoter sequence was transcriptionally active by testing its ability to drive the expression of a P*yuk-lacZ* reporter construct integrated at an ectopic chromosomal locus (*amyE*::P*yuk-lacZ*). Chromosomal DNA isolated from the prototrophic laboratory strain PY79 was used as a template for all PCR amplification. All constructs were first introduced into PY79 by transformation (Gryczan 1978) and then transferred to the 3610 background using SPP1-mediated generalized phage transduction (Yasbin 1974). The bacterial strains used in this study are listed in Supplemental Table 2-1.

Generation of complementing constructs

To generate complementing constructs, the gene of interest was amplified by PCR with primers designed to add *amyE* front and *amyE* back sequences for chromosomal integration, as well as restriction sites for cloning. The reverse oligo also added a C-terminal Myc-tag (protein sequence: MAEQKLISEEDLA) *yukD*, *yukC*, *yukBA* and *yueC* complementing constructs; *yukE* and *yueB* remained untagged. Amplified sequences were cloned by restriction digest into the

integrating plasmid pamyE::Phyperspank, which contains an IPTG-inducible $P_{hyperspank}$ promoter (a gift from David Rudner). This plasmid was integrated into PY79 by double crossover recombination, resulting in the integration of the plasmid at the *amyE* locus in the *B. subtilis* chromosome. All constructs were confirmed by sequencing.

YukE polyclonal antibody generation

A histidine-tagged verison of full-length YukE was utilized for antibody production. *yukE* was PCR-amplified using genomic DNA from the wild-type laboratory strain PY79 as a template. The sequence was inserted into an inducible *E.coli* expression vector and transformed into *E. coli* BL21 cells. YukE-HIS was purified from *E .coli* extracts by affinity chromatography. A rabbit polyclonal serum was raised against this protein (Covance).

SDS-PAGE and immunoblot analysis

Prior to analysis, samples were reduced with 100 mM dithiothreitol (DTT) for 1 hour at 37^{o}C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis. Membranes were probed with anti-YukE (1:1,000) and anti-Myc (1:3,000, ab9106, Abcam, Cambridge, MA). A peroxidase-conjugated goat anti-rabbit secondary antibody (1:3,000, ab6721, Abcam, Cambridge, MA) was detected by chemiluminescence using SuperSignal West Femto (Thermo Scientific). An antibody to the abundant cytosolic protein SigmaA was used to ensure equal protein loading and as a lysis control (1:1,000,000, Fujita 2000). Blots were imaged using a FluorChem FC2 gel documentation system (Alpha Innotech) and provided software.

Mass spectrometry

Bacterial strains were grown in MC media to an OD_{600} of ~2.0. The cells were pelleted and the supernatant was removed to a new conical and filtered through a 0.2 μM filter. Total proteins in

the supernatant were obtained by TCA precipitating 30 mL of sample as described above. The samples were prepared for mass spectrometry analysis as described previously (Garces 2010). Briefly, samples were fractionated by molecular weight on a 10-20% Tricine gel (Invitrogen), followed by in-gel reduction, akylation and trypsin digestion. Samples were run on a Thermo Fisher Scientific LTQ Veloz Mass Spectrometer (Thermo Fisher Scientific, Cambridge, MA). Samples were injected onto a Proxeon Easy nLC system configured with a 5 cm x 100 µm trap packed with 15-20 µm PS-DVB 300A media, and a 25 cm x 100 µm ID resolving column packed with 200A C18AQ media. Buffer A was 96% water, 4% methanol, and 0.2% formic acid. Buffer B was 10 % water, 10% isopropanol, 80% acetonitrile, and 0.2% formic acid; loading buffer (sample loading/rinsing buffer) was 96% water, 4% methanol, and 0.2% formic acid. Samples were loaded at 5 µL a min for 9 min, and a gradient from 0-60% B at 375 nl/min was run over 70 min, for a total run time of 115 min (including regeneration and sample loading). Injection standards (Michrom Medium Molecule test mix, 5 angios, and the TP4 peptides) were injected at 61 fmoles per sample. Velos was run in a data dependent 15 configuration, with a full scan run in the in enhance scan mode (3^e4 target), with up to 15MS2 events. Rejection of +1 ions was used in precursor ion selection.

Resulting spectra were searched against a composite database which contained the predicted open reading frames annotated in the genome of *Bacillus subtilis* 168 supplemented with common contaminates using SEQUEST (Thermo Scientific, San Jose, CA). Peptides were filtered at a 1% FDR with PeptideProphet and grouped into proteins with ProteinProphet (Keller 2005) with a cutoff of 0.95. Spectral counts across the gel slices for three biological replicates were pooled, and then levels of protein expression between strains were compared using an extended G-test (Zhang 2006). Data was corrected for multiple testing (Benjamini and Hochberg) using a p value of ≤ 0.01; for a given protein, a criterion of having ≥ 5 peptides in at least one strain was set.

β-galactosidase assays

β-galactosidase assays were performed as previously described (Camp 2009). Briefly, bacterial strains were grown in the specified media at 37°C. 1 mL of cells were harvested at the given time point and the OD600 was recorded. Cell pellets were resuspended in 0.5 mL Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl, 38 mM β-mercaptoethanol) supplemented with 0.2 mg/ml lysozyme in a clear well 96-well plate. After incubation for 20 minutes at 37°C, reactions were started by adding 50 μL of 4 mg/mL ONPG (2-nitrophenyl β-D-galactopyranoside) (Sigma-Aldrich). The OD420 values of the samples were recorded once per minute for sixty minutes at 37°C in a Synergy 2 plate reader (BioTek). β-galactosidase activity corresponds to the rate of ONPG conversion, and it is calculated as follows: arbitrary units (AU) = [slope of OD420 * dilution factor]/[OD600 of harvested cells].

Assays of biofilm and pellicle formation

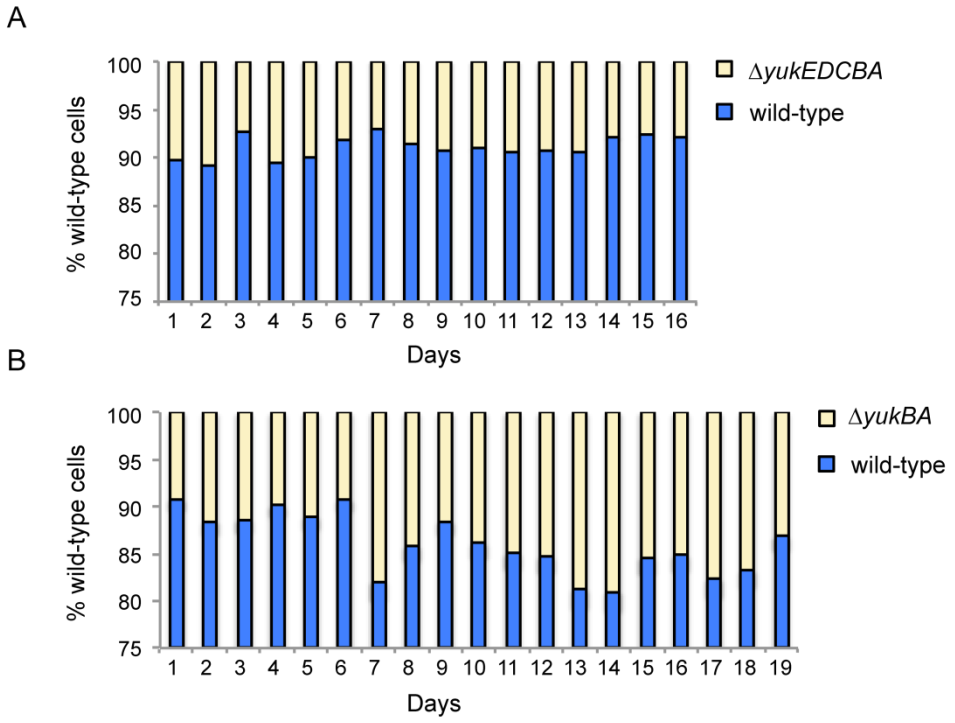
For colony architecture analysis on solid media, a fresh colony was resuspended in 30 μL of PBS; 2 μL was spotted onto an MSgg plate with 1.5% Bacto agar (Branda 2001) and incubated at 30°C for 3 days. Biofilms were imaged using a Leica Wild M10 camera with a Planapo 0,63x objective.

Competition assay

A wild-type strain containing an inducible *lacZ* reporter was co-cultured with one of the *yuk* deletion strains in either LB or MC media (starter culture). The culture was placed rolling at 22°C for 24 hours and the OD600 was recorded. Mixed populations were created with the desired starting ratio of mutant:wild-type strain. The mixed population was diluted 1:1000 to start a new 5 mL culture; culture was placed rolling at 22°C for 24 hours. The following day, dilutions of the mixed population were plated in triplicate onto LB plates supplemented with IPTG (1 mM) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.02% wt/vol) and

placed at 37°C for 12-15 hours. The wild-type blue colonies and mutant white colonies were counted and the numbers were recorded. Every 24 hours, the culture was used to inoculate a new culture (1:1000 dilution) and was plated for colony counts. This 24-hour process was repeated until the ratio of wild-type to mutant strain stabilized.

2.5 Supplementary Materials



Supplemental Figure 2-1. *yuk* deletion strains do not have a competitive growth defect as compared to the wild-type strain.

(A). and (B). To assess the fitness of each strain, Δ*yukEDCBA* (A) and Δ*yukBA* (B) deletion strains were grown in competition with a wild-type PY79 strain containing an inducible *lacZ* reporter. Cultures were started at a ratio of 10% mutant to 90% wild-type cells. On the indicated day, the percentage of each strain within a culture was determined by plating on LB plates supplemented with IPTG and X-gal and counting the resulting blue (wild-type) and white (mutant) colonies. Shown are the mean percentages averaged from triplicate platings for each day.

Strain	Genotype[A]	Source/Reference
PY79	Prototrophic domesticated laboratory strain	Youngman 1983
3610	Undomesticated wild strain	Branda 2001
bLH015	yukE::erm-Pyuk	This Chapter
bLH018	yukEDCBA::erm-Pyuk	This Chapter
bLH019	amyE::Pyuk-lacZ	This Chapter
bLH021	ΩPyuk-lacZ	This Chapter
bLH109	yueB::erm	This Chapter
bLH110	yukBA::erm-Pyuk	This Chapter
bLH274	3610 yukE::erm-Pyuk	This Chapter
bLH278	3610 yukBA::erm-Pyuk	This Chapter
bLH285	3610 amyE::Pyuk-lacZ	This Chapter
bLH286	3610 ΩPyuk-lacZ	This Chapter
bLH288	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-myc	This Chapter
bLH404	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-myc	This Chapter
bLH421	yukD::erm-Pyuk	This Chapter
bLH422	yukC::erm-Pyuk	This Chapter
bLH458	yukD::erm-Pyuk, amyE::Phyperspank-yukD-myc	This Chapter
bLH497	3610 yueB::erm	This Chapter
bLH500	yukC::erm-Pyuk, amyE::Phyperspank-yukC-myc	This Chapter
bLH505	3610 yukD::erm-Pyuk	This Chapter
bLH506	3610 yukC::erm-Pyuk	This Chapter
bLH510	3610 yukD::erm-Pyuk, amyE::Phyperspank-yukD-myc	This Chapter
bLH511	3610 yukC::erm-Pyuk, amyE::Phyperspank-yukC-myc	This Chapter
bLH532	yueB::erm, amyE::Phyperspank-yueB-yueC-myc	This Chapter
bLH533	yukE::erm-Pyuk, amyE::Phyperspank-yukE	This Chapter

Supplemental Table 2-1. Strains used in this study.

[A] All strains are isogenic with the *B. subtilis* laboratory wild-type strain PY79 unless otherwise indicated; "3610" indicates that the strain is isogenic with the *B. subtilis* undomesticated wild-type strain 3610.

Chapter 3: Powering ESX secretion

Chapter 3A: The *B. subtilis* ATPase YukBA requires rotational symmetry among enzymatic domains for function

3A.1 Introduction

AAA+ family ATPases are molecular machines which couple the energy derived from ATP hydrolysis to perform diverse functions within the cell, including the assembly, operation, or disassembly of protein complexes, protein unfolding and degradation, and the translocation and secretion of macromolecules (Neuwald 1999, Ogura 2001, Maurizi 2001). The AAA+ ATPase domain is highly conserved from eukaryotes to prokaryotes, despite the wide range of functions performed by enzymes of this superfamily. The structure of many AAA+ proteins has been solved, revealing a strong preference for these proteins to complex as a hexameric ring (Neuwald 1999, Ogura 2001). However, we have a limited understanding of the mechanism by which these protein complexes couple ATP hydrolysis to the mechanical movement of substrates. Thus, it is not yet clear whether each AAA+ protein has a unique mechanical mechanism by which ATP hydrolysis powers work or whether general rules apply.

Despite their common hexameric form, the manner in which the individual ATPase subunits of these enzymes assemble and coordinate nucleotide hydrolysis varies. The composition of a hexameric machine can either be homeogenous, formed by six identical protein subunits, or heterogenous, where nonidentical proteins come together to form a hexamer. In some homohexameric enzymes, ATP is symmetrically bound to each enzymatic subunit. This is often suggestive of a concerted mechanism of ATP firing, in which all six subunits bind and hydrolyze ATP simultaneously (Gai 2004). Alternatively, for some homohexamers, only a subset of the six potential active sites can bind nucleotide simultaneously. Upon binding, these subunits undergo a conformational change, which generates structural asymmetry within the enzyme. While each subunit within the hexamer is biochemically equivalent in its ability to bind and hydrolyze ATP, this conformational asymmetry dictates the pattern of active site firing within the ring. Structural asymmetry often correlates with mechanistic models involving asynchronous patterns of ATP

firing. These include a sequential model, in which individual subunits hydrolyse ATP in turn around the hexameric ring, and a probabilistic model, where individual subunits fire independently and in a random order (Martin 2005). The heterogeneous subunit composition of heterohexameric enzymes allows for the evolution of subunit specialization, where active sites can perform nonidentical functions. Additionally, subunits can be differentially required for enzyme function, serving active, regulatory, or structural roles. The ability to identify the correct mode of action for a particular ATPase is central to uncovering how the AAA+ machine uses ATP hydrolysis to complete the task at hand.

The mechanism of ATP firing for a given enzyme is typically determined by testing the functional consequences of mixing active and inactive subunits in varying ratios. However, for a homohexameric enzyme, the results from such experiments are often difficult to interpret, as this approach does not fix the arrangement or relative contributions of active and inactive subunits within the hexamer. To overcome this obstacle, investigators have used a strategy of covalently linking some or all of the subunits within a hexamer to create covalent multimers (Martin 2005, Crozat 2010). Inactive subunits can be introduced into these multimers in defined geometric arrangements to determine the biochemical and spatial requirements for enzyme function.

Missing from these mechanistic studies are the molecular motors responsible for protein secretion from bacterial cells. One interesting group of proteins in which to study the mechanics of ATPase activity are the ATPases of ESX-type secretion systems of Gram-positive bacteria, also known as type VII secretion systems (Abdallah 2007). ESX-type secretion systems are found throughout actinobacteria, firmicutes and chloroflexi, but the best studied is the ESX-1 system of *Mycobacterium tuberculosis* (Mtb), as this secretion system is required for the virulence of this clinically relevant human pathogen (Gey van Pittus 2001, Pallen 2002, Stanley 2003, Guinn 2004). Interestingly, the ATPases of ESX systems have a domain structure unique to this class of

proteins, where each protein contains two or three ATPase domains. This is reminiscent of the linked subunit studies described above, thereby creating a unique opportunity to study the mechanism of these ATPases using a natural linked subunit model. By understanding the functional consequences of inactivating subunits in a variety of geometric arrangements we can better understand the mechanism by which ESX-associated ATPases use ATP hydrolysis to power protein translocation.

To address more detailed mechanistic questions about the function and mechanism of action of ESX-associated ATPases, we have developed a model system using the ESX-type secretion system of *Bacillus subtilis* (*B. subtilis*). We leverage the *B. subtilis* model to assess complex formation and domain requirements of the ESX-associated ATPase, YukBA. While the majority of studies addressing the molecular mechanisms of ATPases have used truncated, purified protein subunits to reconstitute hexameric complexes *in vitro*, we test the ATPase requirements of native YukBA. Therefore, each enzymatic domain of YukBA is in a fixed orientation not only in relation to the other domains within a complex, but also relative to the cell membrane and other components of the secretion apparatus. In this system, we find that YukBA dimerizes to form a hexamer of enzymatic subunits that show differential ATPase requirements for protein secretion. Interestingly, we find a requirement for rotational symmetry in the nucleotide binding/hydrolysis activity of subunits that has not been previously described in the study of other ATPases.

3A.2 Results

Domain structure of ESX-encoded ATPases

One defining feature of all ESX loci is the presence of an FtsK/SpoIIIE family ATPase. In contrast to the canonical members of this protein family, which have a single ATPase domain, ESX-associated ATPases possess multiple (2-3) nucleotide binding domains. In fact, among

ATPases of the FtsK/SpoIIIE family, the presence of multiple enzymatic domains is unique to ESX-associated ATPases (Pallen 2002, Figure 3A-1A). Studies of ESX-type systems from multiple organisms have demonstrated that these locus-encoded ATPases are required for the proper functioning of the secretion system, as determined by the secretion of the conserved EsxA-like substrate protein (Stanley 2003, Burts 2005, Akpe San Roman 2010).

The *B. subtilis* ESX-type secretion system is encoded within a five gene operon, currently termed the *yuk* operon, which includes the FtsK/SpoIIIE family ATPase YukBA and the EsxA homologue YukE (São-José 2004). (In the original annotation of the *B. subtilis* genome a sequencing error caused *yukB* and *yukA* to be annotated as two separate open reading frames. Upon correction, this single gene was named *yukBA* (São-José 2004)). YukBA contains three ATPase domains, each identifiable by a Walker A motif (GXXXXGKT/S), the conserved nucleotide binding motif common to all P-loop NTPases (Walker 1982, Saraste 1990) (Figure 3A-1A). The conserved lysine (K) residues of these three motifs are located at amino acids K688, K1016 and K1299 of the YukBA protein. We have recently demonstrated that the *yuk* locus operates as a canonical ESX-type secretion system, where each gene within the operon is required for the secretion of YukE (Huppert et al., in preparation). Therefore, we use YukE secretion as an assay for YukBA function (Figure 3A-1B).

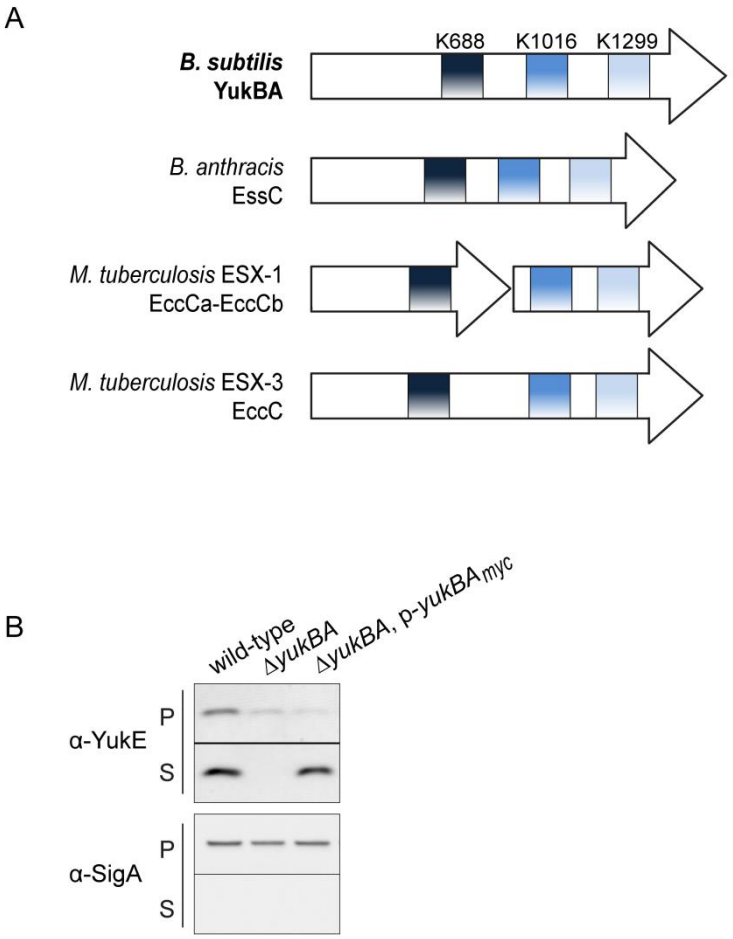


Figure 3A-1. ESX-encoded ATPases.
(A). Protein domain structure of the ESX-encoded ATPases of *B. subtilis*, *B. anthrasis* and *M. tuberculosis* ESX-1 and ESX-3 loci. ATPase domains are indicated by blue boxes. Amino acid position of the *B. subtilis* YukBA Walker A motif active site lysine of each ATPase domain is indicated. (B). Immunoblot demonstrating the requirement for YukBA for YukE secretion. Cell pellet (P) and culture supernatant (S) samples were separated by SDS-PAGE under reducing conditions and analyzed by immunoblot with an α-YukE antibody. An antibody to SigmaA was used as a lysis and loading control.

YukBA is a dimer

Crystal structures of many AAA+ family ATPases have shown that these enzymes assemble as homo- or heterohexameric ring-shaped oligomers. Likewise, FtsK, the canonical FtsK/SpoIIIE family ATPase, functions as a hexameric ring made up of six identical protein subunits (Massey

2006). However, it was unclear how the multiple nucleotide-binding domains of ESX-associated ATPases would assemble. We postulated that these proteins might dimerize, to create a functional hexameric complex containing six ATPase domains, or could form a homohexameric protein complex.

To address this question, we defined the oligomeric state of the ESX-associated ATPase YukBA of *B. subtilis* using gel-exclusion chromatography to assess the size of the native protein complex. As YukBA is a 171 kDa integral membrane protein, we isolated a crude membrane fraction from *B. subtilis* ectopically expressing a functional copy of YukBA-Myc and then solubilized membrane proteins with the non-ionic detergent dodecyl-β-D-maltoside (DDM). The clarified detergent-soluble membrane fraction was resolved on a Sephacryl S-300 column; fractions were analyzed by SDS-PAGE, followed by immunoblot analysis with anti-Myc antibodies. In order obtain solublized YukBA-Myc, we expressed this protein in a variety of bacterial strain backgrounds. When expressed in *B. subtilis* Δ*yukE*, we identified a single peak of YukBA-Myc eluting at a predicted molecular weight of ~450-380 kDa (Figure 3A-2). This molecular weight is consistent with that of a YukBA dimer, corrected for the predicted molecular weight of a DDM micelle (70 kDa) (Linke 2009). These results suggest that YukBA dimerizes to create a functional complex containing six ATPase domains. We hypothesize that this complex is functionally equivalent to the ring-shaped hexamers formed by AAA+ family ATPases. Interestingly, in wild-type cells we could not resolve the size of YukBA, as the protein eluted only in the void volume (data not shown). While we cannot rule out technical explanation for these findings, it is possible that in the presence of substrate a higher order protein complex is formed.

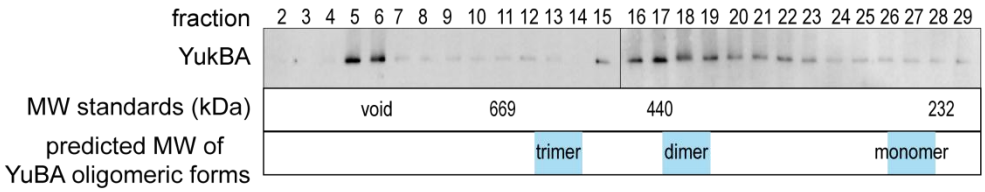


Figure 3A-2. YukBA is a dimer.
Immunoblot of eluted fractions containing YukBA-Myc. The detergent solublized membrane fraction from a Δ*yukE*, *yukBA-myc* strain was applied to a Sephacryl S-300 gel filtration column. Eluted fractions were concentrated by TCA precipitation, separated on an SDS-PAGE gel under reducing conditions, and analyzed by immunoblot with α-Myc antibodies. YukBA-Myc is detected in fractions corresponding to a molecular weight between ~450-380 kDa. Fraction number and elution profile of reference proteins are indicated (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa). Bottom panel indicates predicted molecular weight of YukBA oligomeric forms (accounting for the size of an associated DDM micelle).

The ATPase domains of YukBA are differentially required for YukE secretion

Heterohexamers, by definition, are composed of nonidentical enzymatic subunits. The six active sites of the oligomeric enzyme can contribute equally to function, or they can be functionally nonequivalent. Therefore, we sought to determine the requirements for each of the three structurally distinct active sites within the YukBA dimer. To do so, we inactivated each domain, both singly and in combination, by mutating the conserved lysine (K) residue to an alanine (A). This mutation has previously been shown to prevent ATP binding, thereby rendering the enzymatic activity of the ATPase domain nonfunctional (Omote 1992, Stephens 1995). We then tested the ability of each mutant protein to complement *B. subtilis* Δ*yukBA* for YukE secretion. As seen in Figure 3A-3, the introduction of a wild-type copy of YukBA into Δ*yukBA* successfully restores YukE secretion. When the most N-terminal ATPase domain of YukBA is mutated, YukBA^{K688A}, the expression of this protein fails to complement Δ*yukBA* for YukE secretion (Figure 3A-3A). This is true when this domain is mutated singly or in any combination (*yukBA*^{K688AK1016A}, *yukBA*^{K688AK1299A}, or *yukBA*^{K688AK1016AK1299A}). Therefore, the YukBA^{K688} nucleotide binding domain is fully required for YukE secretion. In contrast, when YukBA^{K1016} or

YukBA^{K1299} are mutated, these single mutant proteins are fully able to restore YukE secretion in Δ*yukBA*. Likewise, when the YukBA^{K1016AK1299A} double mutant protein is expressed in the Δ*yukBA* strain, this protein is fully able to restore YukE secretion. These results demonstrate that YukBA^{K1016} and YukBA^{K1299} are not required for YukE secretion, neither individually or in a redundant fashion. From these results we can conclude that the three ATPase domains of YukBA are differentially required for secretion, as the activity of only the most N-terminal domain, YukBA^{K688}, is required for secretion.

From these results, we cannot distinguish whether the requirement of YukBA^{K688} is at the level of ATP binding or ATP hydrolysis, as the lysine (K) to alanine (A) mutation is known to prevent ATP binding and therefore ATP hydrolysis cannot occur. To address the possibility that YukE secretion may require nucleotide binding, we also made lysine (K) to threonine (T) mutations within each Walker A motif of YukBA, as this mutation has been shown in some systems to allow ATP binding but prevent ATP hydrolysis. We found each YukBA K to T mutant strain to have the same phenotype as the corresponding K to A mutant strain, in regards to the ability to complement Δ*yukBA* for secretion (Figure 3A-3B). Though these results do not suggest distinct requirements for ATP binding versus hydrolysis, *in vitro* ATPase assays will be necessary to verify the nucleotide binding properties of each YukBA mutant.

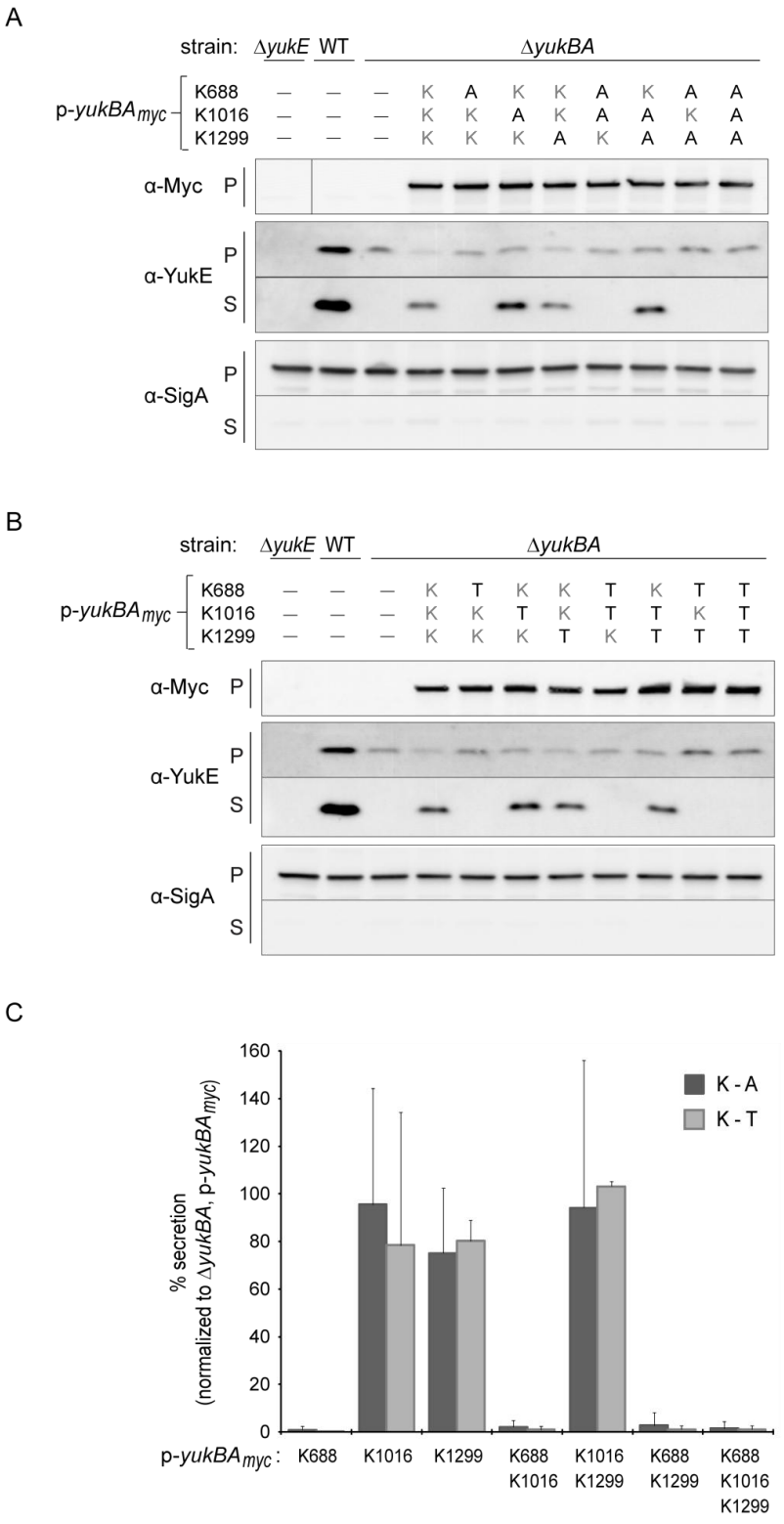


Figure 3A-3. YukBA^{K688} is required for YukE secretion.

Figure 3A-3 (continued). (A). and (B). Immunoblot analysis of cell pellet (P) and culture supernatant (S) of indicated strains grown in LB media. Each *yukBA* Walker A motif mutant was C-terminally *myc*-tagged, fused to an IPTG-inducible promoter ($P_{hyperspank}$), and inserted in single copy at a nonessential locus in the chromosome of the Δ*yukBA* strain. YukBA K to A (A) and K to T (B) Walker A motif mutants were assayed. Reduced P and S samples were separated on an SDS-PAGE gel and analyzed by immunoblot with YukE-specific antibodies. An α-Myc antibody was used to verify expression of the YukBA complementing constructs; an antibody to SigmaA was used as a lysis and loading control. (C). YukE secretion was quantitated through densitometry of the YukE-specific band in the supernatant of each strain. The level of YukE secretion is presented as the percentage of secretion in each Δ*yukBA* strain complemented with a *yukBA* Walker A mutant allele as compared to a Δ*yukBA* strain complemented with wild-type *yukBA*. Data represent the mean + standard deviation of 4 biologic replicates (K to A strains) or 2 biologic replicates (K to T strains).

Each ATPase domain of YukBA is functional and dominant

In addition to providing energy to power substrate translocation, the enzymatic activity of ATPases can be required for the proper assembly or stability of protein complexes involving components of the secretion machine. One way to distinguish between these possible functions is to assess whether an ATPase mutant protein displays functional dominance when expressed in the presence of a wild-type copy of the protein. A dominant phenotype suggests that the mutant protein is able to enter into a complex with wild-type protein, but cannot provide the energy required for substrate translocation, thereby rendering the complex nonfunctional. If dominance is not seen, it suggests that the mutant protein is not able to enter into complexes with wild-type proteins and ATPase activity may be required for complex formation.

To assess whether the enzymatic activity of $YukBA^{K688}$ is required for complex formation or substrate translocation, we created a merodiploid strain ectopically expressing the $YukBA^{K688A}$ mutant in an otherwise wild-type *B. subtilis* strain and assayed YukE secretion. Analysis of the culture supernatant of the $YukBA^{K688A}$ expressing merodiploid strain showed a >80% reduction in YukE secretion (Figure 3A-4A). Likewise, when $yukBA^{K688A}$ is mutated in combination with either other Walker A motif ($yukBA^{K688AK1016A}$, $yukBA^{K688AK1299A}$, or $yukBA^{K688AK1016AK1299A}$), we see

dramatically reduced YukE secretion as compared to the wild-type control. We quantitated the level of YukE in the supernatant of each mutant strain as compared to the wild-type merodiploid strain expressing wild-type YukBA (Figure 3A-4C). These results suggest that the enzymatic activity of YukBA^{K688} is not required for complex formation, but two functional YukBA^{K688} domains are required for secretion. In agreement with our previous findings, we did not see significant differences in dominance between strains where the Walker A motif lysine (K) of YukBA was mutated to an alanine (A) versus a threonine (T) (Figure 3A-4B).

Surprisingly, when YukBA^{K1016} or YukBA^{K1299} was mutated, individually or in combination, these mutants show a dominant effect on YukE secretion (Figure 3A-4A/B). These results are independent of whether the Walker A motif lysine (K) was mutated to alanine (A) or threonine (T). Therefore, though these domains are not required for secretion, they are functional and play a role that requires the ability to bind and/or hydrolyze ATP. Most interestingly, these data reveal a requirement for rotational symmetry in the nucleotide binding state between domains across the hexamer (Figure 3A-5C).

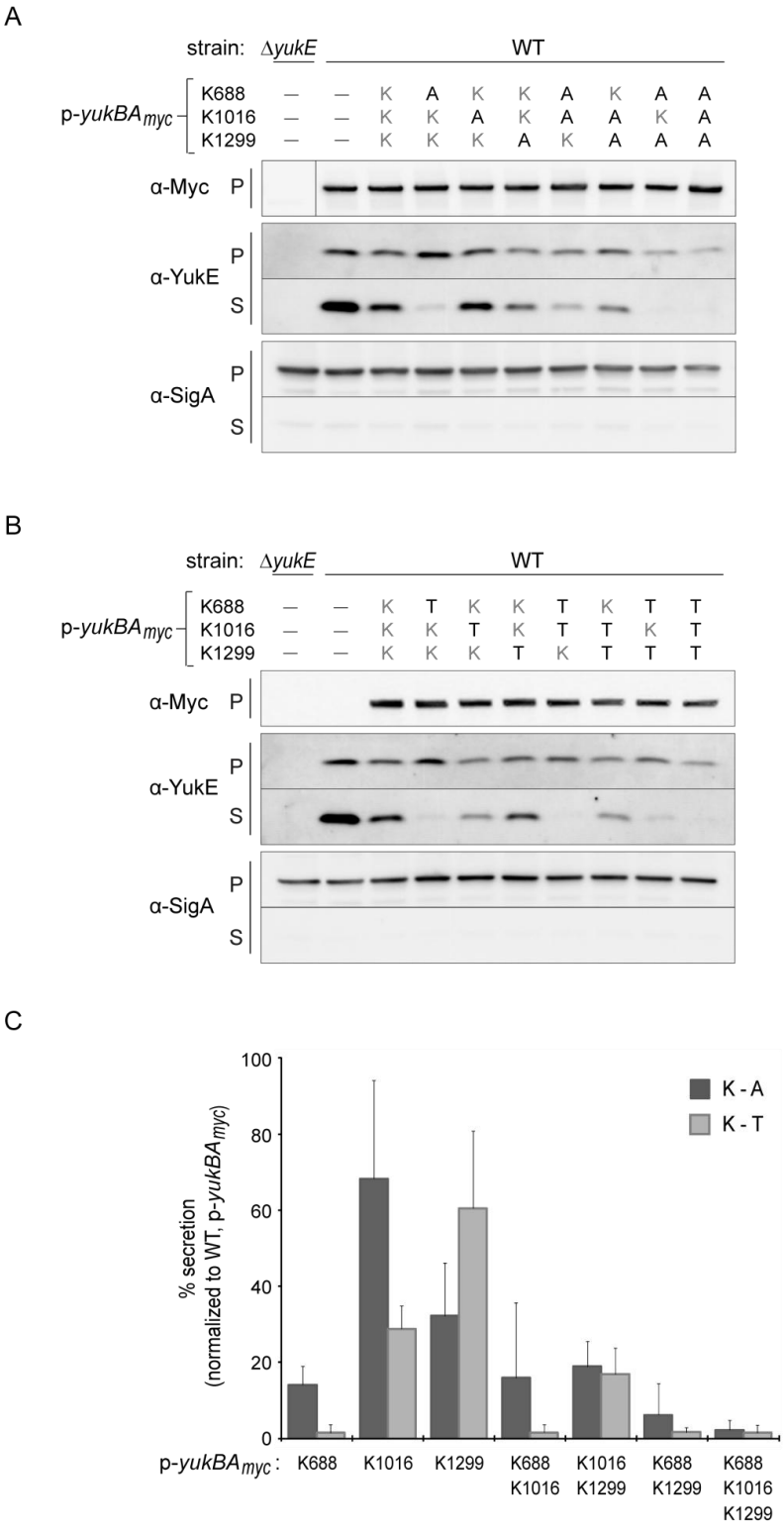


Figure 3A-4. Each YukBA Walker A motif mutant is dominant over wild-type.

Figure 3A-4 (continued). (A). and (B). Immunoblot analysis of cell pellet (P) and culture supernatant (S) of indicated strains grown in LB media. Each *yukBA* ATPase mutant gene was C-terminally *myc*-tagged, fused to an IPTG- inducible promoter ($P_{hyperspank}$), and inserted in single copy at a nonessential locus in the chromosome of an otherwise wild-type strain. YukBA K to A (A) and K to T (B) ATPase mutants were assayed. Reduced P and S samples were separated on an SDS-PAGE gel and analyzed by immunoblot with YukE-specific antibodies. An α-Myc antibody was used to verify expression of the YukBA complementing constructs, and an antibody to SigmaA was used as a lysis and loading control. (C). YukE secretion was quantitated through densitometry of the YukE-specific band in the supernatant of each strain. The level of YukE secretion is presented as the percentage of secretion in each *yukBA* mutant-expressing merodiploid strain as compared to a wild-type strain expressing wild-type *yukBA*. Data represent the mean + standard deviation of 4 biologic replicates (K to A strains) or 2 biologic replicates (K to T strains).

3A.3 Discussion

The FtsK/SpoIIIE family ATPases of ESX-type secretion systems uniquely contain multiple ATPase domains. This is a distinguishing feature of this class of enzymes, but the structural and functional consequences of this domain organization are unknown. We propose that the native conformation of YukBA is a dimer, thereby forming a complex with six ATPase domains. The simplest model is that YukBA forms a head-to-tail dimeric ring to produce a functional hexamer. This configuration, as opposed to a head-to-head interaction, most easily ensures that the same face of each domain is oriented toward the inner surface of the hexameric ring upon dimerization (Figure3A-5B).

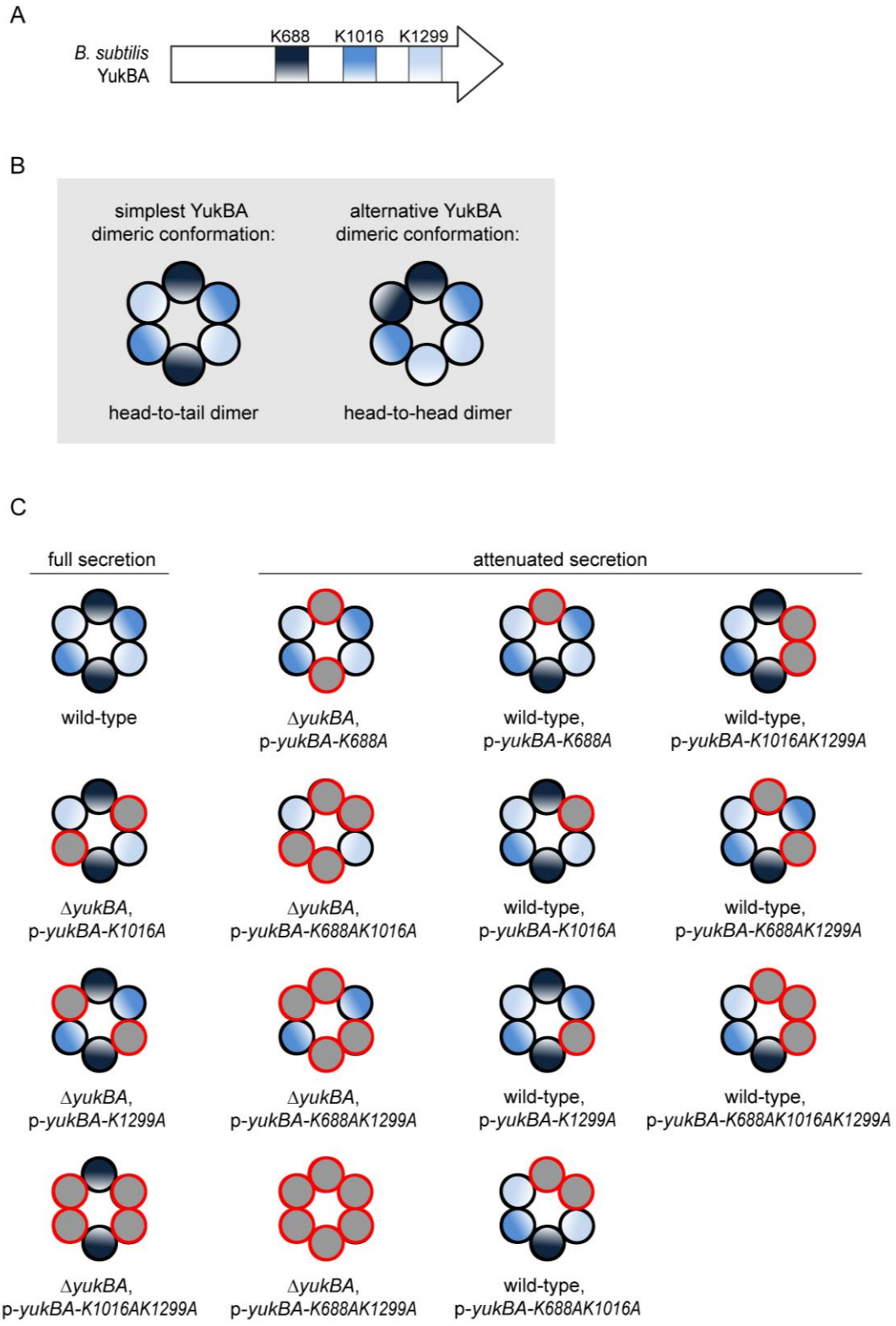


Figure 3A-5. Models demonstrating the functional requirements of YukBA.

Figure 3A-5 (continued). (A). Protein domain structure of YukBA. ATPase domains are indicated by blue boxes; amino acid position of each Walker A motif active site lysine is indicated. (B). Two possible conformations of a YukBA dimer. A head-to-tail configuration ensures that the same face of each domain is orientated toward the inner surface of the ring. (C). Secretion phenotype of dimers formed by YukBA single, double, and triple Walker A motif mutant proteins, in the absence or presence of wild-type YukBA. Domains shaded grey and outlined in red represent domains that have been rendered inactive through mutation of the Walker A motif active site lysine (K).

The mechanism by which a hexameric ATPase couples the energy from ATP hydrolysis to the mechanical movement necessary for substrate translocation is poorly understood for most enzymes. There are currently three primary models used to describe the firing pattern among the subunits of a hexamer: concerted, sequential, and probabilistic. The six subunits of an enzyme can equally contribute to function, or the subunits within an enzyme can be differentially required for function. The inherent domain structure of ESX ATPases provides a natural linked subunit model, allowing us to introduce mutations in known geometric arrangements and test the functional consequences on protein secretion. Here, we find that the six ATPase domains within a YukBA dimer are differentially required for YukE secretion. ATP binding/hydrolysis by most N-terminal domain, YukBA^{K688}, is absolutely required, while YukBA^{K1016} and YukBA^{K1299} are not required for YukE secretion. These results are inconsistent with a model requiring all six domains within a hexamer to fire synchronously, such as the all subunit concerted model. The data also rule out other models in which all six ATPase domains must fire in order to power secretion, including an obligate sequential model. Additionally, our data exclude a probabilistic firing pattern, because in this model, as currently conceived, all subunits are equivalent and any subunit can be rendered inactive without consequence to overall enzyme function, as long as the minimum number of active subunits is retained. Each of the above models of ATPase firing can be edited to describe the firing pattern between only a subset of subunits within a hexamer, the designated active subunits. For example, ATP hydrolysis by the F1-ATPase, which is composed of alternating catalytic and noncatalytic subunits, occurs in a defined rotary sequence amongst the

three active subunits (Boyer 1993). This mechanism of action has been designated a three-site sequential model. Likewise, in our system, the two YukBA^{K688} domains may fire simultaneously, or alternatively, they may fire sequentially or completely independent of one another. Currently, we cannot distinguish between these possibilities.

Using a test of dominance we find that the YukBA^{K1016} and YukBA^{K1299} domains are functional, but not required for YukE secretion. Interestingly, YukBA function requires rotational symmetry within a dimeric complex, such that the opposing subunits within the ring are in the same phase as one another (Figure 3A-5C). For example, when the two YukBA^{K1016} domains within a hexamer are both wild-type or are both mutated, such that they are in the same nucleotide binding state as one another, the resulting YukBA dimer is functional and YukE is secreted at wild-type levels. But, when the two YukBA^{K1016} domains within a dimer are out of phase with each other, as is the case upon dimerization of one wild-type YukBA protein with one YukBA^{K1016A} mutant protein, the complex is no longer able to function. Likewise, the YukBA^{K1299} Walker A motif shows this same requirement for rotational symmetry.

We envision a model in which the YukBA dimer is anchored at the cell membrane through the N-terminal transmembrane domains of each monomer. Upon substrate interaction, the two YukBA^{K688} domains act as the primary catalytic subunits, coupling the hydrolysis of ATP to move YukE across the cell membrane. The remaining four active sites within the dimer, YukBA^{K1016} and YukBA^{K1299} from each monomer, may act in a regulatory capacity to coordinate the firing pattern of the catalytic subunits. For secretion to occur, each domain must be in register with the active site across the hexameric ring, demonstrating a strict requirement for intersubunit communication for function.

3A.4 Materials and Methods

Culture of *B. subtilis* and preparation of whole cell pellets and culture supernatants

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman 1983). Strains were maintained in Miller LB broth (Acros, New Jersey, USA) or on Difco Miller LB agar (BD, Sparks, MD). When appropriate, antibiotics were included in the growth media as follows: 100 µg/mL spectinomycin, 5 µg/mL chloramphenicol, 5 µg/mL kanamycin, 10 µg/mL tetracycline, and 1 µg/mL erythromycin plus 25 µg/mL lincomycin (mls). Pellet and supernatant samples were grown and processed as described in Chapter 2.4.

Strain construction and generation of complementing constructs

General methods for molecular cloning and strain construction were performed according to published protocols (Sambrook 2006). Generation of p-*yukBA-myc* complementing construct was described in Chapter 2.4. The bacterial strains used in this study are listed in Supplemental Table 3A-1.

Site directed mutagenesis of *yukBA* expression vectors

The active site lysine of each Walker A motif of YukBA was mutated to an alanine or threoinine using QuikChange site directed mutagenesis (Stratagene). All mutations were introduced into the integrating plasmid p-amyE::hyperspank-*yukBA-myc*. Double and triple mutant constructs were made through sequential mutation. The following QuikChange primer pairs were used (mutated bases in bold): *yukBAK688A* (forward primer: 5' GGACAACGGGATCAGGG**GCG**AGTGAATTTCTCCAGACC 3'; reverse primer: 5' GGTCTGGAGAAATTCACT**CGC**CCCTGATCCCGTTGTCC 3'), *yukBAK688T* (forward primer: 5' GGACAACGGGATCAGGG**ACG**AGTGAATTTCTCCAGACC 3'; reverse primer: 5' GGTCTGGAGAAATTCACT**CGT**CCCTGATCCCGTTGTCC 3'), *yukBAK1016A* (forward

primer: 5’ GCTCTTCGGGCTACGGA**GCG**TCGATTGCAGCCGCAACG 3’; reverse primer: 5’ CGTTGCGGCTGCAATCGA**CGC**TCCGTAGCCCGAAGAGC 3’), *yukBAK1016T* (forward primer: 5’ GCTCTTCGGGCTACGGA**ACG**TCGATTGCAGCCGCAACG 3’; reverse primer: 5’ CGTTGCGGCTGCAATCGA**CGT**TCCGTAGCCCGAAGAGC 3’), *yukBAK1299A* (forward primer: 5’ GCCAAACTCAGCGCGGG**GCG**ACAAACGTCCTAAAAGTC 3’; reverse primer: 5’ GACTTTTAGGACGTTTGT**CGC**CCCGCGCTGAGTTTGGC 3’), *yukBAK1299T* (forward primer: 5’ GCCAAACTCAGCGCGGG**ACG**ACAAACGTCCTAAAAGTC 3’; reverse primer: 5’ GACTTTTAGGACGTTTGT**CGT**CCCGCGCTGAGTTTGGC 3’). All constructs were confirmed by sequencing.

Preparation of detergent solublized membrane fractions

B. subtilis cultures started from a single colony were normalized to an OD600 of 0.02 in LB media (50 mL cultures) and grown 2 hours at 37^{o}C, at which time 100 μM IPTG was added to induce protein expression; cultures were grown an additional 2 hours. Cell pellets were harvested by centrifugation and washed two times with 1X SMM (0.5 M Sucrose, 20 mM $MgCl_2$, 20 mM Maleic acid pH 6.5) at room temperature. Cells were resuspended in 10 mL 1x SMM and protoplasted with 1mg/ml lysozyme. Protoplasts were collected by centrifugation and flash frozen in liquid N_2. Thawed protoplasts were disrupted by osmotic lysis with 6 mL hypotonic buffer (20 mM HEPES pH 8.0, 200 mM NaCl, protease inhibitor cocktail). $MgCl_2$ and $CaCl_2$ were added to 1 mM, and lysates were treated with 10 μg/ml DNase I and 20 μg/ml RNase A for 1 hour on ice. The membrane fraction was separated by centrifugation at 100,000 *g* for 1 hour at 4^{o}C. Crude membranes were flash frozen in N_2, resuspended in 2.4 mL buffer (20 mM HEPES pH 8.0, 200 mM NaCl, protease inhibitor) and solublized by the addition of the detergent DDM (n-dodecyl-B-d-maltopyranoside, Calbiochem, La Jolla, CA) to a final concentration of 1%. This mixture was incubated at 4^{o}C for 1 hour. Membrane soluble and insoluble fractions were

separated by centrifugation at 100,000 *g* for 1 hr at 4°C.

Sizing by FPLC

The membrane soluble fraction was diluted 10-fold in buffer (20 mM Hepes pH 8.0, 200 mM NaCl) to give a final DDM concentration of 0.1%. Sample was spun at 100,000 *g* for 1 hr at 4°C to remove any precipitate. 0.5 mL sample was loaded onto a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare) equilibrated with buffer (20 mM Hepes pH 8.0, 200 mM NaCl, 0.1% DDM). 1 mL fractions were collected and concentrated by precipitation with 10% TCA, resuspended in SDS sample buffer (6x, nonreducing SDS sample buffer, Boston BioProducts), and analyzed by immunoblot. Reference proteins used for calibration were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) (HMW Gel Filtration Calibration Kit, Amersham).

SDS-PAGE and immunoblot analysis

Prior to analysis, samples were reduced with 100 mM DTT for 1 hour at 37°C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis. Membranes were probed with anti-YukE (1:1,000) and anti-Myc (1:3,000, ab9106, Abcam, Cambridge, MA). A peroxidase-conjugated goat anti-rabbit secondary antibody (1:3,000, ab6721, Abcam, Cambridge, MA) was detected by chemiluminescence using SuperSignal West Femto (Thermo Scientific). An antibody to the abundant, cytosolic protein SigmaA was used to ensure equal protein loading and as a lysis control (1:1,000,000, Fujita 2000). Blots were imaged and densitometric quantitation of YukE secretion was performed using a FluorChem FC2 gel documentation system (Alpha Innotech) and provided software.

3A.5 Supplementary Materials

Strain	Genotype[A]	Source/Reference
PY79	Prototrophic domesticated laboratory strain	Youngman 1983
bLH015	yukE::erm-Pyuk	Chapter 2
bLH110	yukBA::erm-Pyuk	Chapter 2
bLH266	amyE::Phyperspank-yukBA-myc	This Chapter
bLH267	amyE::Phyperspank-yukBA-K688A-myc	This Chapter
bLH268	amyE::Phyperspank-yukBA-K1016A-myc	This Chapter
bLH269	amyE::Phyperspank-yukBA-K1299A-myc	This Chapter
bLH318	amyE::Phyperspank-yukBA-K688AK1016A-myc	This Chapter
bLH319	amyE::Phyperspank-yukBA-K688AK1299A-myc	This Chapter
bLH320	amyE::Phyperspank-yukBA-K1016AK1299A-myc	This Chapter
bLH321	amyE::Phyperspank-yukBA-K688AK1016AK1299A-myc	This Chapter
bLH397	amyE::Phyperspank-yukBA-K688T-myc	This Chapter
bLH398	amyE::Phyperspank-yukBA-K1016T-myc	This Chapter
bLH399	amyE::Phyperspank-yukBA-K1299T-myc	This Chapter
bLH400	amyE::Phyperspank-yukBA-K688TK1016T-myc	This Chapter
bLH401	amyE::Phyperspank-yukBA-K688TK1299T-myc	This Chapter
bLH402	amyE::Phyperspank-yukBA-K1016TK1299T-myc	This Chapter
bLH403	amyE::Phyperspank-yukBA-K688TK1016TK1299T-myc	This Chapter
bLH404	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-myc	Chapter 2
bLH405	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688A-myc	This Chapter
bLH406	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1016A-myc	This Chapter
bLH407	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1299A-myc	This Chapter
bLH408	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688AK1016A-myc	This Chapter
bLH409	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1016AK1299A-myc	This Chapter
bLH410	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688AK1299A-myc	This Chapter
bLH411	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688AK1016AK1299A-myc	This Chapter
bLH412	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688T-myc	This Chapter
bLH413	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1016T-myc	This Chapter
bLH414	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1299T-myc	This Chapter
bLH415	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688TK1016T-myc	This Chapter
bLH416	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K1016TK1299T-myc	This Chapter
bLH417	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688TK1299T-myc	This Chapter
bLH418	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-K688TK1016TK1299T-myc	This Chapter

Supplemental Table 3A-1. Strains used in this study.

[A] All strains are isogenic with the *B. subtilis* laboratory wild-type strain PY79 unless otherwise indicated; "3610" indicates that the strain is isogenic with the *B. subtilis* undomesticated wild-type strain 3610.

Chapter 3B: EccCa-EccCb ATPase requirements for ESX-1 secretory activity

3B.1 Introduction

The ESX-1 secretion system of *Mycobacterium tuberculosis* (Mtb) was the first identified ESX-type secretion system, and it has been shown to be a major virulence determinant of this human pathogen. The Mtb ESX-1 secretion system is encoded within a 20 gene locus responsible for the secretion of six protein substrates: EsxA, EsxB, EspA, EspB, EspC, and EspR (Sørensen 1995, Berthet 1998, Fortune 2005, MacGurn 2005, Xu 2007, McLaughlin 2007, Raghavan 2008). While many genes within the locus have been shown to be required for functional secretion, the mechanism by which apparatus components assemble and translocate these substrates is poorly understood. Through the study of discrete components of this system, we and others aim to better understand the mechanism by which this alternative protein secretion system functions and mediates pathogenesis.

Within every genetic locus encoding for an ESX-type secretion system, there is an ATPase containing multiple FtsK/SpoIIIE family ATPase domains (Pallen 2002). In contrast to most ESX-associated ATPases of this family, which are encoded for by a single polypeptide, the ATPase of the Mtb ESX-1 locus was divided into two genes, *eccCa* and *eccCb*, during the evolution of the ESX-1 genetic duplication (Gey van Pittius 2001). *eccCa* contains one ATPase domain, identifiable by a conserved Walker A nucleotide binding motif, while *eccCb* contains two Walker A motifs (Figure 3B-1). The EccCa and EccCb proteins are predicted to work together as a single functional unit to power ESX-1 secretion. In agreement with this prediction, using a yeast-two hybrid system, EccCa and EccCb have been shown to interact *in vitro* (Stanley 2003).

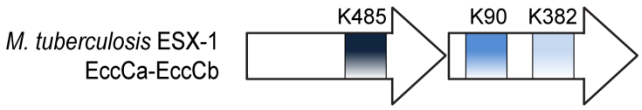


Figure 3B-1. Mtb ESX-1-encoded ATPases EccCa-EccCb.
(A). Protein domain structure of the Mtb ESX-1-encoded ATPases EccCa-EccCb. ATPase domains are indicated by blue boxes; amino acid position of the Walker A motif active site lysine of each ATPase domain is indicated.

To investigate the mechanism by which ESX-1 functions, we have sought to understand the role of the locus-encoded FtsK/SpoIIIE family ATPases *eccCa* and *eccCb* in ESX-1-mediated secretion. Additionally, in Chapter 3A, we demonstrated that only the most N-terminal ATPase domain of the *B. subtilis* ESX-associated ATPase YukBA was required for the function of the *yuk* secretion system, while the second and third domains of this protein play a non-required role. Given the split protein architecture of the Mtb ESX-1-encoded ATPases, we sought to determine if EccCa-EccCb show parallel domain requirements, or if the added complexity of the split protein reveal differential domain requirements between the two systems.

3B.2 Results

Each ATPase domain of EccCa-EccCb is required for ESX-1-mediated secretion

The deletion of *eccCa* or *eccCb* has previously been shown to block ESX-1-mediated secretion (Stanley 2003, Guinn 2004). To verify these findings in our experimental system, we assessed the secretion of known ESX-1 substrates in strains containing a transposon insertion in either *eccCa* (*eccCa*::Tn) or *eccCb* (*eccCb*::Tn). Using immunoblot analysis and protein-specific antibodies, we looked for the expression and secretion of EsxA, EsxB, and EspA in each transposon mutant strain as compared to wild-type H37Rv. As a negative control, we included a ΔRD1 knockout strain, which lacks *esxA* and *esxB*, and has previously been shown not secrete

EspA (Fortune 2005). In agreement with previously published results, the *eccCa*::Tn and *eccCb*::Tn strains do not secrete EsxA, EsxB, or EspA (Figure 3B-2). Expression of *eccCa-eccCb* in tandem from an episomal construct restores secretion in the *eccCa*::Tn mutant strain. (Expression of *eccCa* alone did not restore secretion; it is likely that this is due to a polar effect of the transposon insertion on the downstream e*ccCb* gene). Likewise, episomal expression of *eccCb* restores the secretion of ESX-1 substrates in the *eccCb*::Tn strain.

To test the requirements for functional EccCa and EccCb ATPase activity for ESX-1 secretion, we mutated the conserved lysine (K) in each Walker A motif to an alanine (A) and then tested the ability of each mutant protein to complement ESX-1 secretion in an *eccCa*- or *eccCb*-null strain. The expression of the $EccCa^{K485A}$ ATPase mutant (in tandem with wild-type EccCb) failed to restore the secretion of EsxA, EsxB, and EspA in *eccCa*::Tn (Figure 3B-2). Likewise, each EccCb ATPase mutant protein ($EccCb^{K90A}$, $EccCb^{K382A}$, and the $EccCb^{K90AK382A}$ double mutant) was unable to complement *eccCb*::Tn for secretion. These results demonstrate that the ATPase activity of each individual nucleotide binding domain of EccCa-EccCb is required for ESX-1 secretion.

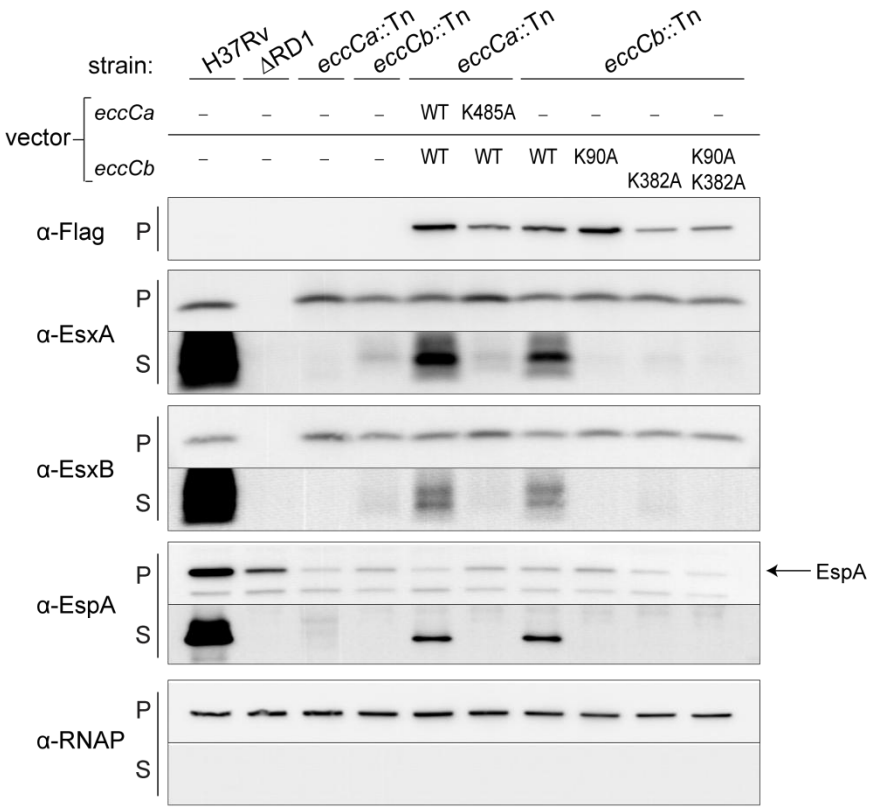


Figure 3B-2. Each ATPase domain of EccCa-EccCb is required for ESX-1-mediated secretion.
Immunoblot analysis of cell pellet (P) and culture supernatant (S) of the indicated strains grown in Sauton's media. Samples were separated by SDS-PAGE under reducing conditions and analyzed for ESX-1 secretion using antibodies specific to the ESX-1 substrates EsxA, EsxB, and EspA. In each complementing construct, *eccCb* was C-terminally *flag*-tagged. An antibody to *E. coli* RNAP was used as a lysis and loading control. Data are representative of at least two independent experiments.

Some EccCa-EccCb ATPase mutants are dominant negative on ESX-1-mediated secretion

Secretion system-associated ATPases can provide energy for various steps in the secretion process, including the recruitment or assembly of secretion apparatus components, as well as substrate translocation. It is possible that the three ATPase domains of EccCa and EccCb are differentially required for these processes. To address whether the enzymatic activity of EccCa and/or EccCb is required to mediate protein-protein interactions, we asked whether the Walker A

motif mutant forms of these proteins show dominance over wild-type. We reasoned that if the ATPase mutant EccCa or EccCb proteins are still able to enter into protein complexes, then their expression in a wild-type background will result in reduced ESX-1 secretion due to the mutant protein interfering with wild-type transport complexes.

To determine whether the EccCa and EccCb Walker A motif mutants display transdominance, we expressed each mutant from an inducible episomal vector in a wild-type strain of Mtb and assayed for functional ESX-1 secretion. We find that the expression of $EccCa^{K485A}$, $EccCb^{K90A}$, or $EccCb^{K382A}$ results in a dominant negative effect on secretion, as demonstrated by the reduced levels of EsxA, EsxB, and EspA in the culture supernatant of these merodiploid strains as compared to wild-type (Figure 3B-3). This phenotype is not simply due to an abundance of EccCa or EccCb within the cell, as overexpression of wild-type copies of these genes does not effect secretion. The transdominant phenotype displayed by the three single EccCa-EccCb ATPase mutants provides strong genetic evidence that these proteins function as part of a protein-protein complex, in accordance with previously published data.

Interestingly, the $EccCb^{K90AK382A}$ double mutant protein shows a significant relief in the dominance phenotype as compared to the single EccCb mutants (Figure 3B-3). While the level of secretion in this strain is not that of wild-type, it is significantly greater than is seen with the expression of any single mutant. These results suggest that some ATPase activity is required for EccCb to form stable protein complexes. Although an alternative explanation of these results is that there is a gross structural malformation of the $EccCb^{K90AK382A}$ protein, this is not likely as a lysine (K) to alanine (A) substitution is known to be structurally conservative and immunoblot analysis of the cell pellet shows that the $EccCb^{K90AK382A}$ mutant protein is both expressed and stable.

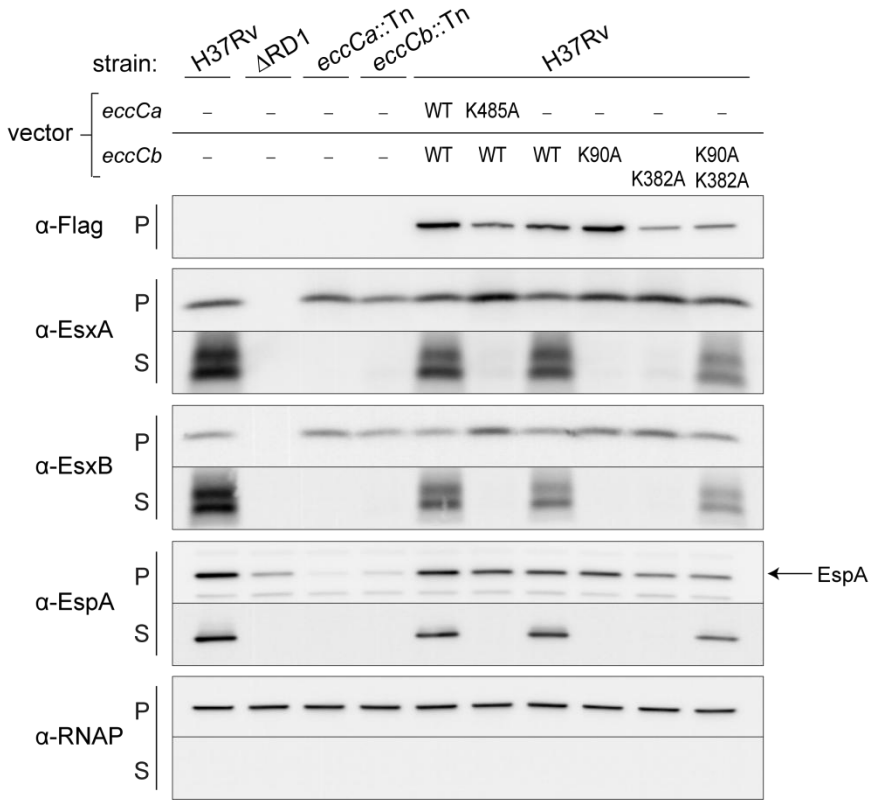


Figure 3B-3. Some ATPase domains of EccCa-EccCb are dominant over wild-type. Immunoblot analysis of cell pellet (P) and culture supernatant (S) of the indicated strains grown in Sauton's media. Samples were separated by SDS-PAGE under reducing conditions and analyzed for ESX-1 secretion using antibodies specific to the ESX-1 substrates EsxA, EsxB, and EspA. In each complementing construct, *eccCb* was C-terminally *flag*-tagged. An antibody to *E. coli* RNAP was used as a lysis and loading control. Data are representative of at least three independent experiments.

3B.3 Discussion

The ESX-associated FtsK/SpoIIIE family ATPases uniquely contain multiple enzymatic domains. While the ESX-1 genes *eccCa* and *eccCb* encode two polypeptides, genetic and biochemical evidence suggest that these proteins act together as a single functional unit, thereby forming a complex with a total of three ATPase domains. We find that each ATPase domain is individually required for ESX-1-mediated secretion. Additionally, we provide strong genetic evidence that

EccCa and EccCb function as part of a protein complex, either with each other and/or other components of the secretion system. Interestingly, EccCb-mediated dominance is relieved upon mutation of both active sites within the protein ($EccCb^{K90AK382A}$), suggesting that some energy from ATP binding or hydrolysis is required to either form or stabilize EccCb-containing complexes.

In Chapter 3A we demonstrate that the *B. subtilis* ESX-associated ATPase YukBA forms a dimeric complex and we propose that the six ATPase domains of this YukBA dimer form a hexameric ring of active sites, as is common to many AAA+ ATPases. We hypothesize that this structural assembly is conserved among ESX-associated ATPases, and that in the Mtb ESX-1 system, two EccCa and two EccCb proteins interact to form and function as a hexameric ring containing six ATPase domains.

The ESX-1 secretion system of Mtb and the ESX-type *yuk* secretion system of *B. subtilis* show differential energetic requirements for substrate secretion. While each ATPase domain within EccCa-EccCb is required for secretion, only the most N-terminal ATPase domain of YukBA is required for functional secretion. We hypothesize that, given the like requirement for the most N-terminal domain of each enzyme ($EccCa^{K485}$ and $YukBA^{K688}$), these domains represents a function common to both systems. Given that our data suggests that this domain is not required for these proteins to enter into protein-protein complexes, we propose that the energy derived from ATP hydrolysis by $EccCa^{K485}$ and $YukBA^{K688}$ is directly coupled to substrate translocation.

One possible explanation for the additional requirement for the enzymatic activity of the two ATPase domains of EccCb is that, due to the split architecture of EccCa and EccCb, energy is required to mediate the interaction between these two proteins. In this model, having one functional EccCb ATPase domain is sufficient to produce a functional complex, but without the

energy derived from ATP hydrolysis from either of these domains, the interaction between EccCa and EccCb is too weak to form a stable, ring-shaped complex. Additionally, EccCb ATPase activity may be used to recruit substrates or regulate the interaction of substrates with the pore of the secretion machine to control the timing and specificity of secretion. These activities could represent Mtb-specific requirements, for example, to either direct the secretion of multiple substrates or to tightly regulate secretion upon interaction with a host cell.

Finally, we previously demonstrated that although the enzymatic activity of YukBA^{K1016} and YukBA^{K1299} of *B. subtilis* is not required for YukE secretion, these domains must be in the same nucleotide binding state as the corresponding domain across the hexameric ring. We predict that the EccCa-EccCb dimeric ring also requires this radial symmetry, but our ability to see this requirement is overshadowed by the requirement for these domains to mediate the interaction with EccCa.

3B.4 Materials and Methods

Culture of Mtb and preparation of whole cell pellets and culture supernatants

Mtb (H37Rv) strains were maintained in Middlebrook 7H9 media supplemented with oleic acid-albumin-dextrose-catalase (OADC, Difco) and 0.05% Tween 80. The generation of H37RvΔRD1 (Lewis 2003) and the identification and confirmation of the Rv3870::Tn (*eccCa*::Tn) and Rv3871::Tn (*eccCb*::Tn) transposon mutant strains have been previously described (Guinn 2004).

For analysis of protein expression and secretion, bacterial cultures grown to mid-log phase were normalized to an OD600 of 0.3 in fresh 7H9 media supplemented with OADC, 0.05% Tween 80 and 100 ng/ml anhydrotetracycline (AT, Spectrum Chemicals, Gardena, CA), when appropriate. Cultures were grown overnight at 37^{o}C to induce gene expression of the complementing construct

24 hours prior to beginning culture supernatant collections. Cultures were pelleted, washed, resuspended in Sauton's media supplemented with 0.05% Tween 80 and AT and grown for 48 hours at 37°C. Cell pellets were collected by centrifugation, resuspended in protein extraction buffer (50 mM Tris·HCL pH 7.5, 5 mM EDTA, protease inhibitor cocktail) and disrupted by bead beating. SDS sample buffer (Novex 2x Tricine SDS sample buffer, Invitrogen) was added and samples were heated at 95°C for 20 minutes before removal from the biosafety level 3 facility. Protease inhibitor cocktail was added to the culture supernatants, which were then sterilized by double filtration through 0.2 μM filters. The culture supernatants were concentrated by precipitation with 10% TCA, resuspended in SDS sample buffer and heated to 95°C for 20 minutes.

Generation of complementing constructs

To generate Mtb complementing constructs, the genomic region encoding *eccCa*, *eccCb*, or *eccCa-eccCb* in tandem (including the native intergenic region), was amplified from H37Rv genomic DNA (*eccCa* forward primer: GGCTAAGAAGGAGATATACATATGACGACCAAGAAGTTCACT; *eccCa* reverse primer: CTTGTCGTCGTCGTCCTTGTAGTCCGGTGTCGGCGCCTCCTC; *eccCb* forward primer: GGCTAAGAAGGAGATATACATATGACTGCTGAACCGGAAGTA; *eccCb* reverse primer: CTTGTCGTCGTCGTCCTTGTAGTCACCGGCGCTTGGGGGTGC). Forward primers added a ribosomal binding site (GAAGGAGATATACAT) upstream of each start codon, and the reverse oligo of *eccCb* added a C-terminal Flag-tag (protein sequence: DYKDDDDK). A second round of PCR added *att* sites for Gateway recombination of the product and the PCR products were recombined into a Gateway donor vector (pDONR, Invitrogen, Carlsbad, CA) and transferred to an episomal expression vector (pTET), which allows for regulated gene expression under the control of a tetracycline inducible mycobacterial promoter (Ehrt 2005). All constructs were confirmed by sequencing. The resulting pTET constructs or an empty vector were

transformed into wild-type H37Rv, *eccCa*::Tn and *eccCb*::Tn strains. These strains were maintained under selection with 50 μg/ml Hygromycin (Roche, Mannheim, Germany) to prevent plasmid loss.

Site directed mutagenesis of *eccCa* and *eccCb* expression vectors

The *eccCaK485A* mutation was introduced into pTET-*eccCa-eccCb-flag* using QuikChange site-directed mutagenesis (Stratagene) (mutated bases in bold) (forward primer: 5' GGACCACGGGGTCTGGG**GCG**TCCGAATTCCTGCGCAC 3'; reverse primer: 5' GTGCGCAGGAATTCGGA**CGC**CCCAGACCCCGTGGTCC 3'). The *eccCbK90A* mutation was introduced via PCR mutagenesis and cloning of an internal gene fragment. The fragment was amplified with a reverse primer that mutated lysine 90 to an alanine (forward primer: 5' GGCTAAGAAGGAGATATACATATGACTGCTGAACCGGAAGTA 3'; reverse primer: 5' CCATCGTCTGCAGTAGCGTCGA**CGC**CCCGGTTTGAGGTGCGCC 3'), digested with SacI and PstI and ligated into vector pDONR-*eccCb-flag*, which had also been digested with SacI and PstI, to create p*DONR- eccCbK90A-flag*. The construct was then transferred pTET. The *eccCbK382A* mutation was introduced into pTET-*eccCb-flag* using QuikChange site-directed mutagenesis (Stratagene) (forward primer: 5' CTACTGATCTTCGGTGCGGCCAAATCGGGC**GCG**ACGACCATTGCCCACGCGATAGCG CGCGCC 3'; reverse primer: 5' GGCGCGCGCTATCGCGTGGGCAATGGTCGT**CGC**GCCCGATTTGGCCGCACCGAAGAT CAGTAG 3'). The *eccCbK90AK382A*mutant was made through the introduction of the K90A mutation into pTET-*eccCbK382A-flag* as described above. All constructs were confirmed by sequencing.

SDS-PAGE and immunoblot analysis

Prior to analysis, samples were reduced with 100 mM DTT for 1 hour at 37^{o}C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis. Membranes were probed with anti-EsxA (ESAT-6, 1:2,000, ab26246, Abcam, Cambridge, MA), anti-EsxB (CFP-10, 1:1,000, ab45074, Abcam, Cambridge, MA), anti-EspA (1:2,000, Fortune 2005) and anti-Flag (1:5,000, F7425, Sigma, Saint Louis, MO). Peroxidase-conjugated goat anti-rabbit (Abcam, Cambridge, MA) and goat anti-mouse (32430, Thermo Scientific) secondary antibodies were used at a concentration of 1:40,000 and 1:1,000, respectively. Secondary antibodies were detected by chemiluminescence using SuperSignal West Femto (Thermo Scientific). An antibody to *E. coli* RNAP was used to ensure equal protein loading and as a lysis control (1:1,000, WP023, Neoclone). Equal loading of culture supernatants was confirmed by visualizing the total protein loaded by Coomassie staining.

Chapter 4: Energetic requirements of ESX-mediated cell wall functions

4.1 Introduction

The ESX-1 secretion system is a critical mediator of virulence for the intracellular pathogen *Mycobacterium tuberculosis* (Mtb). Despite the importance of this system, its primary function and the mechanism by which this system mediates virulence is unclear. This alternative secretion system has historically been thought to function in a manner homologous to the type III or type IV secretion systems of Gram-negative bacteria, which secrete virulence effectors directly into the host cell environment, resulting in the modulation of the host cell for the benefit of the bacterium. While this is an attractive model, the active translocation of the known ESX-1 substrate proteins directly into the host cell cytoplasm has not been convincingly demonstrated. Additionally, none of the ESX-1 substrates contain functional domains common to known virulence effector proteins.

Currently, the only ESX-1 substrate for which an *in vivo* virulence activity has been proposed is EsxA. EsxA is hypothesized to possess membranolytic activity, resulting in the formation of pores in host cell membranes. This model stems from data by Hsu et al. who used an artificial membrane system to show that purified EsxA could disrupt a lipid bilayer (2003). Also using an *in vitro* system, de Jonge et al. demonstrate a strong association between purified EsxA and liposomes, which results in the destabilization and lysis of the liposomes (2007). While the physiological relevance of the above *in vitro* systems can be questioned, additional studies have provided indirect evidence in support of ESX-1-mediated host cell cytolysis. For example, strains containing deletions in ESX-1 showed a failure to lyse cells in culture (Hsu 2003, Guinn 2004) and a loss of cell-to-cell spread *in vitro* (Gao 2004, Guinn 2004). In mouse models of infection, ESX-1-deficient strains showed less necrosis (Junqueira-Kipnis 2006) and a lack of tissue invasiveness and lung damage, presumably due to a loss of bacterial egress from infected cells, resulting in reduce spreading (Hsu 2003). While these studies demonstrate an association

between ESX-1-mediated secretion and various cytolytic pathologies, it is unclear whether these effects are primary or secondary to ESX-1 function. To date, the pore-forming ability of EsxA remains controversial.

More recently, an alternative ESX-1 function was demonstrated when Garces et al. found ESX-1 to be a critical mediator of bacterial cell wall integrity (2010). These results validate a long ago recognized connection between colony morphology, often a gross readout of cell wall composition, virulence, and ESX-1 (Calmette 1927, Steenken 1934, Pym 2002). Using a structure-function based approach, it was demonstrated that ESX-1-mediated secretion could be dissociated from virulence; a strain expressing a point mutant of the substrate EspA was fully competent for the secretion of all known ESX-1 substrates, but was severely attenuated for virulence in macrophages and mice. Most interestingly, the attenuation of this strain correlated with a loss of cell wall integrity, as determined by increased susceptibility to the detergent SDS, a known cell wall stress. Strains lacking the ESX-1 locus or the ATPase *eccCb* were also more susceptible to detergent stress. These data suggest that the primary target of ESX-1 is the mycobacterial cell wall, and that it is through this function that ESX-1 mediates virulence.

The extent to which these two separable ESX-1 functions are connected is not known. To begin to address this question, we sought to compare the energy requirements of secretion versus modulation of cell wall integrity. Additionally, questions remain as to the *primary* function of ESX-1, and ESX-type systems more broadly. Much of the controversy surrounding the model that the primary mechanism of ESX-1-associated virulence is mediated by the pore-forming ability of EsxA stems from the conservation of ESX-loci and EsxA in nonpathogenic bacterial species. Therefore, we also sought to investigate whether ESX-1-mediated cell wall integrity may represent a general function of ESX-type secretion systems. Using our *B. subtilis* model

system, we sought to test the effect of *yuk* operon mutations on bacterial cell wall integrity and biofilm formation.

4.2 Results

Mtb ESX-1-mediated cell wall integrity is dependent upon EccCb ATPase activity

Prior to secretion, a regulated sequence of events must occur. The structural components of the secretion apparatus must localize and assemble at the cell membrane, and likewise, the substrates must be recruited to this site. In the case of a secretion system with multiple substrates, there must be a mechanism of substrate identification and discrimination. Additionally, proteins often need to be released from a chaperone and/or unfolded prior to entrance into the translocation pore. Upon the completion of these steps, the substrate can be translocated across the cell membrane and/or cell wall to its final destination. Given our knowledge of the ESX-1 system, we can predict many of the prerequisite steps for secretion, but the steps and events required for ESX-1 to ensure cell wall integrity are much less clear. It is known that ATPases can provide the energy to mediate any number of the aforementioned steps leading to secretion. Therefore, we sought to compare the energy requirements of ESX-1-mediated secretion versus cell wall integrity to gain insight into the connection between these two ESX-1 functions.

We hypothesize that the three ATPase domains of EccCa and EccCb may be differentially required for the translocation of protein substrates versus maintaining the integrity of the mycobacterial cell wall. In Chapter 3B, we demonstrated that each ATPase domain within EccCa and EccCb is required for ESX-1-mediated secretion. To test the requirements for EccCa-EccCb enzymatic activity in ensuring cell wall integrity, we assayed the effect of mutating the ATPase domains of these proteins on the ability of the bacterium to survive cell wall stress.

It has previously been shown that EccCb is required for cell survival under SDS-induced stress, and this cell wall defect could be complemented by restoring a wild-type copy of the gene (Garces 2010). To determine whether the ATPase activity of EccCb is required for cell wall integrity, we tested the ability of Walker A mutant forms of this protein to restore the ability of the *eccCb*::Tn strain to survive SDS treatment. We found that neither $EccCb^{K90A}$ nor $EccCb^{K382A}$ was able to complement the *eccCb*::Tn mutant for survival under SDS-induced stress (Figure 4-1). Similarly, when each single mutant protein was introduced into wild-type H37Rv, both resulting merodiploid strains showed an increase in SDS susceptibility as compared to wild-type. Thus, $eccCb^{K90}$and $eccCb^{K382}$ are individually required for both cell wall integrity and protein secretion. (For technical reasons, the EccCa Walker A mutant was not assayed in this experiment).

Interestingly, when *eccCb*::Tn was complemented with the *eccCb* double mutant (*eccCb*::Tn, p-$eccCb^{K90AK382A}$) this strain showed wild-type susceptibility to SDS treatment. This restoration of cell wall integrity is in contrast to the inability of this mutant to restore ESX-1 secretion. When expressed in a wild-type background, the double mutant protein was not dominant over wild-type, as demonstrated by the wild-type SDS susceptibility of the H37Rv, p-$eccCb^{K90AK382A}$ merodiploid strain. Similarly, we previously found that the $eccCb^{K90AK382A}$ double mutant was not dominant over wild-type for secretion. Together, these results suggest that the mechanism by which ESX-1 secretes substrates is at least partially separable from the mechanism by which ESX-1 maintains cell wall integrity.

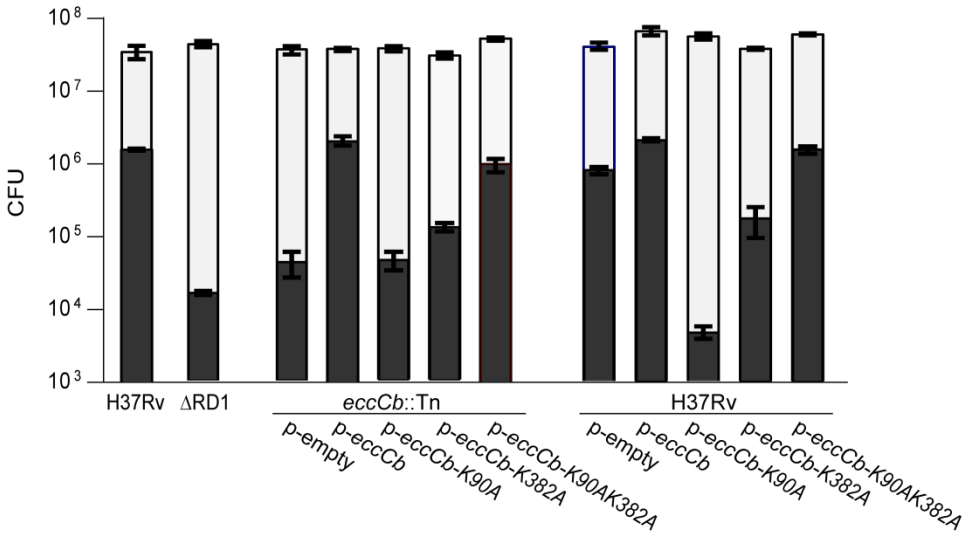


Figure 4-1. EccCb ATPase activity is required for Mtb to survive treatment with SDS. To assess bacterial survival after SDS treatment, the indicated strains were grown overnight in Sauton's media containing 0.1% SDS and then plated for survival (black bars). Untreated bacteria were plated as a control (gray bars). Results represent a single independent culture for each strain, plated for CFU in triplicate. The mean +/- standard deviations of the triplicate platings are expressed.

YueB overexpression by *B. subtilis* leads to increased susceptibility to the antibiotic cefuroxime

ESX-type secretion systems are conserved among both pathogenic and nonpathogenic bacterial species. This includes nonpathogenic mycobacteria, such as the soil-dwelling bacterium *Mycobacterium smegmatis*, as well as various nonmycobacterial Gram-positive species (Gey van Pittius 2001, Pallen 2002). It is currently unclear whether ESX-type systems retain a conserved function among all species. We and others have demonstrated that in multiple bacterial species, ESX-type systems function to secrete EsxA-like proteins, though the functional consequences of this are action unclear. But, the conservation of a cell wall-specific ESX function has not yet been widely tested. Therefore, we sought to further understand the function of the *B. subtilis* ESX-type system by characterizing a variety of phenotypic responses to loss of the *yuk* secretion system.

Given the increased detergent susceptibility demonstrated by Mtb ESX-1 mutants, we began by testing the SDS susceptibility of wild-type, Δ*yukBA*, and Δ*yukBAyueB* strains using a disc plate diffusion assay. At all concentrations of SDS tested, the susceptibilities of the three strains were the same (data not shown).

To more comprehensively screen for phenotypic changes associated with loss of the *yuk* secretion system, phenotypic microarrays of wild-type (PY79), Δ*yukBA*, and Δ*yukBAyueB* strains were performed in conjunction with Biolog (Haywood, CA). Of the thousands of phenotypes tested, very few metabolic or chemical sensitivity tests showed differential phenotypic behavior between these strains. One antibiotic, cefuroxime, showed a mild difference in efficacy against the three strains and warranted further validation. Cefuroxime is a cephalosporin antibiotic which targets the process of bacterial cell wall synthesis. Using a disc plate diffusion assay, we compared the susceptibility of wild-type *B. subtilis* to that of various *yuk* operon mutant strains. All strains tested had a cefuroxime susceptibility equivalent to that of wild-type, except for Δ*yukEDCBA* and Δ*yukBA*, which were more susceptible to cefuroxime (Figure 4-2A). But, the ectopic expression of YukBA in the Δ*yukBA* strain did not complement the susceptibility phenotype of this deletion strain, suggesting that increased susceptibility to cefuroxime is not a YukBA-mediated effect (Figure 4-2B).

Interestingly, in each of the strains demonstrating an increased susceptibility to cefuroxime (Δ*yukEDCBA*, Δ*yukBA*, and Δ*yukBA*, p-*yukBA*), *yueB* is being overexpressed as a result of the method used to generate these deletion strains. As described in Chapter 2, to ensure the expression of downstream operon genes, the native *yuk* promoter (P*yuk*) was reinserted after the antibiotic resistance cassette in each knockout strain. We found that the insertion of P*yuk* directly upstream of *yueB* results in an approximate 2-3 fold increase in *yueB* expression as compared to

wild-type, and when P*yuk* is inserted directly upstream of any other gene within the operon, *yueB* is expressed at wild-type levels (data not shown). Interestingly, the gross colony morphology of the *yueB* overexpressing strains also differ from wild-type, in that the colonies are more mucoid and slightly smaller by visual inspection. This variation in colony morphology is suggestive of cell wall changes in these strains. YueB is a six pass transmembrane protein, and we postulate that increased levels of this protein may alter the cell membrane or cell wall in such a way that increases the cefuroxime susceptibility of the strain. We cannot rule out the possibility that the insertion of the *yuk* promoter at this location affects the expression of another gene which is responsible for the increased susceptibility to cefuroxime phenotype.

To determine whether the increased susceptibility to cefuroxime is specific to this antibiotic, or if instead these strains have a more general cell wall defect, we compared the susceptibility of wild-type and Δ*yukBA* strains to other antibiotics, both those that act on the cell wall and those with other cellular targets. As seen in Figure 4-2C, we did not find a difference in susceptibility of wild-type versus Δ*yukBA* to carbenicillin, kanamycin, vancomycin, chloromphenicol, or tetracycline. Together, these data suggest that the increased expression of *yueB* causes a specific increase in susceptibility to the antibiotic cefuroxime. The mechanism by which YueB may mediate these effects is currently not understood.

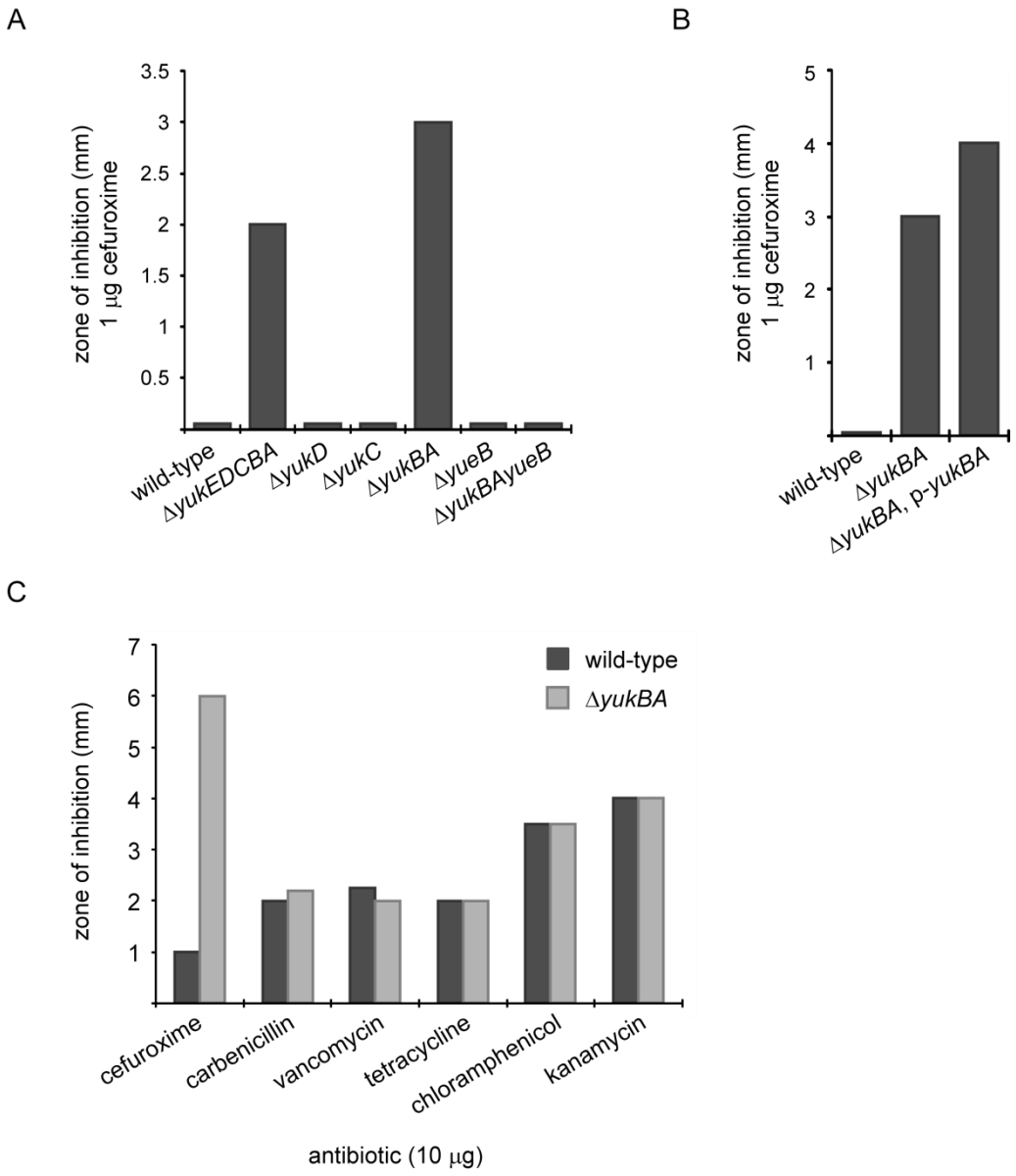


Figure 4-2. Insertion of P*yuk* directly upstream of *yueB* results in increased cefuroxime susceptibility.

(A). Disc plate diffusion assays were used to determine the susceptibility of the indicated strains to cefuroxime. 1 μg of cefuroxime spotted on a paper disc was placed in the center of an LB agar plate on which a lawn of the indicated *B. subtilis* strain had been seeded. After incubation overnight, the zone of growth inhibition surrounding the disc was measured. (B). The disc plate diffusion assay was repeated with wild-type, Δ*yukBA* and Δ*yukBA*, p-*yukBA* strains. IPTG was added to the agar of plates seeded with Δ*yukBA*, p-*yukBA* to induce the expression of the *yukBA* complementing construct. (C). Disc plate diffusion assays were used to test the susceptibility of the wild-type and Δ*yukBA* strains to the indicated antibiotics (10 μg).

***B. subtilis* biofilm formation is dependent upon YukBA ATPase activity**

In Chapter 2, we demonstrate a role for the *yuk* secretion system in biofilm formation by the undomesticated strain *B. subtilis* 3610. Upon deletion of *yukBA*, biofilm architecture is altered from that of wild-type; this phenotype is YukBA-mediated, as complementation of 3610 Δ*yukBA* with a wild-type copy of *yukBA* restores the biofilm to an architecture similar to that of wild-type 3610 (Figure 2-5, Figure 4-3). To begin to understand how the *yuk* locus is involved in biofilm formation, we sought to test whether biofilm formation has the same energetic requirements as YukE secretion. To do so, we tested the ability of the YukBA Walker A motif mutant proteins to restore wild-type biofilm architecture when expressed in a 3610 Δ*yukBA* strain. When the single YukBA mutants are expressed in 3610 Δ*yukBA*, we find that YukBA^{K688A} does not restore biofilm architecture to that of wild-type, while YukBA^{K1016A} and YukBA^{K1299A} are each able to complement the biofilm defect of the *yukBA*-deficient strain (Figure 4-3). These results parallel the energetic requirements for YukE secretion.

When the YukBA ATPase domains are mutated in combination, we find that none of the mutants are able to restore biofilm formation by 3610 Δ*yukBA* to that of wild-type 3610 (Figure 4-3). These results suggest that there may be differential energetic requirements for YukE secretion versus biofilm formation, as complementation with the YukBA^{K1016AK1299A} mutant fully restores YukE secretion, but does not complement biofilm formation. This data supports our previous results demonstrating that the role of the *yuk* system in biofilm formation is independent of YukE secretion. It is possible that biofilm formation requires the secretion of an as of yet unidentified substrate. Although we did not find evidence of additional *yuk* substrates in our mass spec analysis of the culture supernatants of the laboratory strain *B. subtilis* PY79, it is conceivable that secretion is dependent upon growth condition, or that a substrate(s) may be secreted specifically by the *B. subtilis* 3610 strain. Alternatively, the *yuk* system may mediate biofilm formation and/or cell wall composition by a mechanism independent of protein secretion.

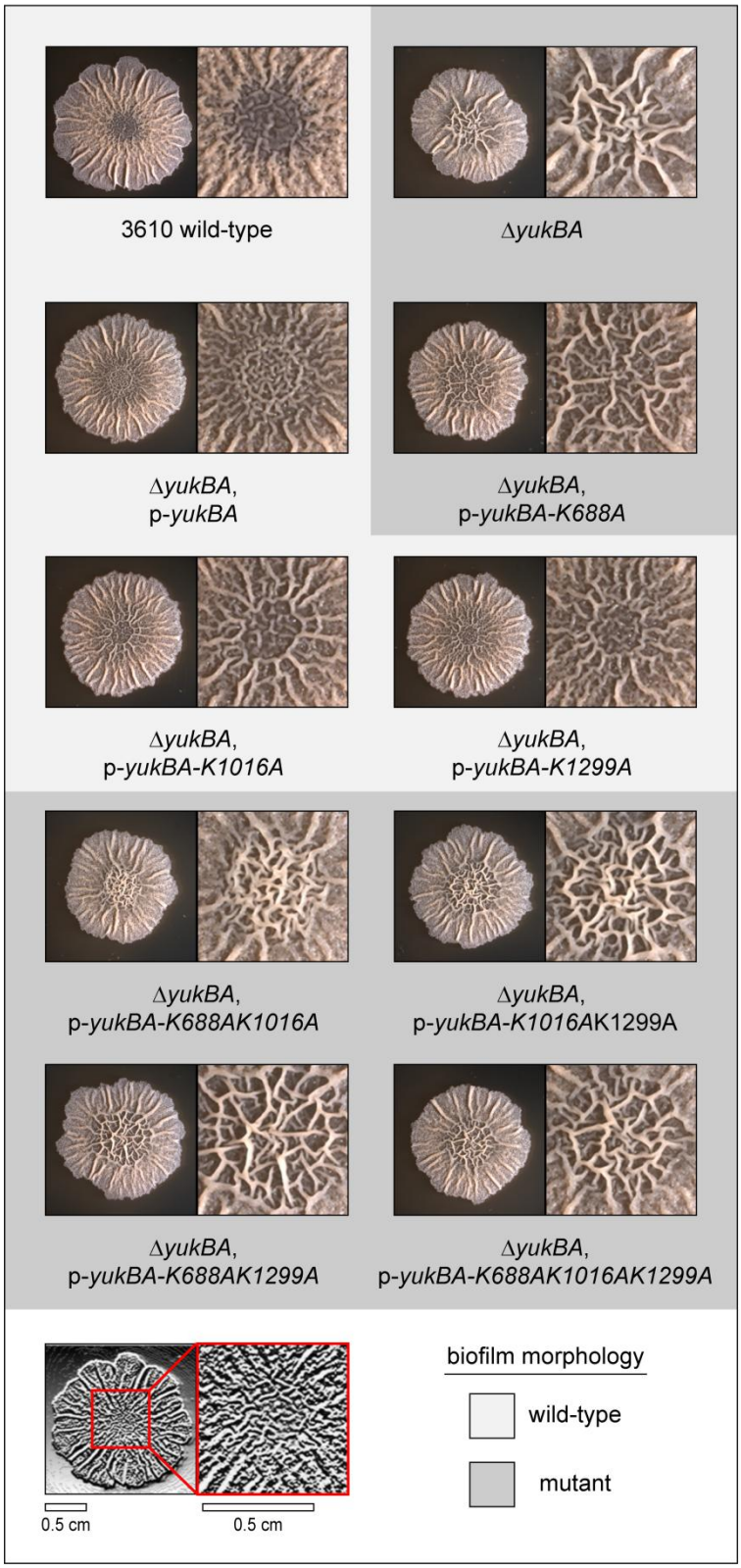


Figure 4-3. YukBA ATPase activity is required for normal biofilm formation.

Figure 4-3 (continued). *B. subtilis* 3610 wild-type, Δ*yukBA*, and Δ*yukBA* strains complemented with YukBA Walker A motif mutants were grown on MSgg agar to induce biofilm growth. Light gray shaded panels indicate a strain which has a wild-type biofilm morphology; dark gray shaded panels indicate a strain demonstrating a mutant biofilm morphology. As diagrammed on the bottom left of the figure, left panels: entire biofilm; right panels: close up view of biofilm center. Scale bar: 0.5 cm, indicated for each view.

4.3 Discussion

The functions of ESX-type secretion systems are still an area of active discovery. Here, we demonstrate partially differential requirements for EccCa-EccCb ATPase activity in ESX-1-dependent cell wall integrity versus ESX-1-mediated secretion. Additionally, we show that the comparable functions of the *yuk* secretion system, YukE secretion, susceptibility to cefuroxime, and normal biofilm formation, also differentially rely on ATPase activity.

Though the requirement of ESX-1 for virulence has historically been attributed to the secretory function of this system, the recent demonstration that protein secretion can be dissociated from virulence highlights the simplicity of this model. The identification of ESX-1 as a critical mediator of bacterial cell wall integrity suggests that the primary target of this system is the bacterium itself. We have found evidence to suggest that ESX-type systems share conserved functions of both protein secretion and an involvement in cell wall formation/maintenance. Though the extent to which these two functions are connected is not yet understood, the differential ATPase requirements for these functions in both Mtb and *B. subtilis* support previous findings that these functions are separable, and suggests that the mechanism by which these systems function in secretion and cell wall integrity differ.

Here, we have demonstrated that the most N-terminal Walker A motif of YukBA, YukBA^{K688}, is required for normal biofilm formation. Additionally, this nucleotide binding site, as well as the

equivalent enzymatic domain in Mtb, EccCa^{K485}, is absolutely required for ESX-mediated secretion. Together, these data suggest that this domain may have a conserved function which is required for both secretion and cell wall related functions.

In Chapters 3A and 3B, we show that the second and third Walker A motifs of EccCa-EccCb and YukBA are differentially required for ESX-mediated secretion. Here, we find that, within both the ESX-1 system and the *yuk* system, these two domains are differentially required for protein secretion versus cell wall related functions. Together, these results suggest that these two domains may have evolved to fulfill both species-specific and function-specific requirements.

4.4 Materials and Methods

Culture of Mtb and *B. subtilis*

Mtb (H37Rv) strains were maintained in Middlebrook 7H9 media supplemented with oleic acid-albumin-dextrose-catalase (OADC, Difco) and 0.05% Tween 80, and when appropriate 50 μg/ml hygromycin. *B. subtilis* PY79 and 3610 strains were maintained in Miller LB broth (Acros, New Jersey, USA) or on Difco Miller LB agar (BD, Sparks, MD). When appropriate, antibiotics were included in the growth media as follows: 100 μg/mL spectinomycin and 1 μg/mL erythromycin plus 25 μg/mL lincomycin (mls).

Strain construction

All strains and complementing constructs (Mtb and *B. subtilis*) were previously described in Chapters 2.4, 3A.4 and 3B.4. *The B. subtilis* strains used in this study are listed in Supplemental Table 4-1.

Mtb detergent susceptibility

SDS susceptibility assays were performed as previously described (Garces 2010). Briefly, strains were grown to early log phase in Sauton's media supplemented with 0.05% Tween 80 and 100 ng/mL AT. Cells were pelleted, washed once and resuspended at a density of 1.26108 cells/mL in 7H9 media containing 0.1% SDS, and 100 ng/mL AT when appropriate. Bacteria were incubated in SDS overnight at 37^{o}C with shaking, washed twice and plated in triplicate for CFU on 7H10 agar plates containing 10% OADC.

***B. subtilis* disc plate diffusion assays**

Mid-exponential phase cultures were evenly plated to seed a lawn on LB agar using glass beads. When appropriate, IPTG was added into the LB agar at a final concentration of 100 μM to induce expression of the complementing construct. Sterile paper discs were placed on the surface of the agar in the center of the petri dish; one disc per plate. 6 μL of the appropriate antibiotic or SDS, diluted to the indicated concentration in sterile ddH_20, was spotted on each disc. When dry, plates were incubated at 37^{o}C overnight.

***B. subtilis* biofilm assays**

For colony architecture analysis on solid media, a fresh colony was resuspended in 30 μL of PBS; 2 μL was spotted onto an MSgg plate with 1.5% Bacto agar (Branda 2001) and incubated at 30°C for 3 days. Biofilms were imaged using a Leica Wild M10 camera with a Planapo 0.63x objective.

4.5 Supplementary Materials

Strain	Genotype[A]	Source/Reference
PY79	Prototrophic domesticated laboratory strain	Youngman 1983
3610	Undomesticated wild strain	Branda 2001
bLH018	yukEDCBA::erm-Pyuk, amyE::kan	Chapter 2
bLH045	yukE::erm-Pyuk, amyE::kan	This Chapter
bLH048	yukEDCBA::erm-Pyuk, amyE::kan	This Chapter
bLH108	yukBAyueB::erm	This Chapter
bLH109	yueB::erm	Chapter 2
bLH110	yukBA::erm-Pyuk	Chapter 2
bLH278	3610 yukBA::erm-Pyuk	Chapter 2
bLH338	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK688AK1016A-myc	This Chapter
bLH340	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK688AK1299A-myc	This Chapter
bLH342	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK1016AK1299A-myc	This Chapter
bLH344	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK688AK1016AK1299A-myc	This Chapter
bLH346	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-myc	This Chapter
bLH348	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK688A-myc	This Chapter
bLH350	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK1016A-myc	This Chapter
bLH352	3610 yukBA::erm-Pyuk, amyE::Phyperspank-yukBAK1299A-myc	This Chapter
bLH404	yukBA::erm-Pyuk, amyE::Phyperspank-yukBA-myc	Chapter 2
bLH421	yukD::erm-Pyuk	Chapter 2
bLH422	yukC::erm-Pyuk	Chapter 2

Supplemental Table 4-1. Strains used in this study.

[A] All strains are isogenic with the *B. subtilis* laboratory wild-type strain PY79 unless otherwise indicated; "3610" indicates that the strain is isogenic with the *B. subtilis* undomesticated wild-type strain 3610.

Chapter 5: Discussion

Despite the importance of the ESX-1 secretion system to Mtb virulence, the primary function of this system and the mechanism by which this system mediates virulence is still unclear. Due to the inherent challenges of working with Mtb, researchers can benefit greatly from the development of model systems in which to address a wide variety of questions regarding Mtb biology. Here, we describe the development of a *B. subtilis* model system in which to study the function and mechanism of action of ESX-type secretion systems.

We have demonstrated that the *yuk* locus encodes a secretion system responsible for the secretion of YukE. Additionally, we have found this system to be important for normal biofilm formation. These findings are reminiscent of the two identified functions of the Mtb ESX-1 system: protein secretion and mediating cell wall integrity. These results suggest that the dual functionality demonstrated by ESX-1 may be a conserved characteristic of ESX-type secretion systems.

ESX-1-mediated cell wall integrity is independent of the secretion of known ESX-1 substrates and interestingly, we have found that the role of the *yuk* system in biofilm formation is independent of YukE secretion. While we have not ruled out the possibility that the *yuk* system mediates biofilm formation through the secretion of an as of yet unidentified protein substrate, these data suggest that ESX-type systems may modulate the bacterial cell surface in a conserved, secretion-independent manner. This may be through the transportation of a nonproteinous substrate(s), such as lipids or small molecules, or ESX systems may act as an important structural scaffold in the cell wall. To further address whether the role of the *yuk* system in biofilm formation is secretion dependent or independent, we propose to screen for additional substrates using the laboratory strain *B. subtilis* PY79 grown in biofilm-inducing conditions, as well as in the undomesticated strain *B. subtilis* 3610.

Here, we have used the *B. subtilis* model system to investigate the molecular mechanism of ESX-associated ATPases. Given the unique multi-FtsK/SpoIIIE domain structure of these ATPases, it was unclear how these enzymes assemble into a functional unit and whether each of the three ATPase domains of EccCa-EccCb and YukBA are functional. We have demonstrated that YukBA forms a dimer, producing a complex with a hexameric ring of enzymatic domains. We hypothesize that this structural configuration is common to ESX-associated ATPases. Additionally, we have demonstrated that each enzymatic domain within these ATPases is functional, though not always required, and that all ATPase domains within the enzyme do not contribute equally to function.

Our data suggest that the most N-terminal Walker A motif of ESX-associated ATPases may share a conserved role acting as the primary source of energy for substrate translocation. The differential requirements for the remaining two ATPase domains of EccCa-EccCb and YukBA suggest that these domains may have evolved to fulfill both species-specific and function-specific requirements.

We hypothesize that the increased energy requirement of ESX-1-mediated secretion reflects the additional complexity in the ESX-1 secretion process as compared to secretion by the *yuk* system. Continued studies are required to test whether this is due to the split protein architecture that unique to ESX-1 and ESX-1-encoded ATPases, or if this additional requirement reflects differences in substrate interactions or regulation between the two systems.

Future studies using the *B. subtilis yuk* system as a model ESX-type system, stand to provide great insight into the mechanism and function of these systems, both in general, and specifically in relation to the role of the ESX-1 system in the pathogenesis of Mtb.

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